UNIVERSITY OF ALBERTA

MECHANISM AND REGULATION OF LIPID TRANSFER PROTEIN

BY

KERRY WOON SING KO ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilmentof the requirements for the degree of **DOCTOR OF PHILOSOPHY**.

IN

MEDICAL SCIENCES - MEDICINE

EDMONTON, ALBERTA

Fall 1966

National Library
of Canada

Acquisitions and
Bibliographic Services Branch

395 Wellington Street
Ottawa, Ontario
K1A 0N4

Bibliothèque nationale
du Canada

Direction des acquisitions et
des services bibliographiques

395, rue Wellington
Ottawa (Ontario)
K1A 0N4

Your file Votre référence

Our file Notre référence

The author has granted an irrevocable non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of his/her thesis by any means and in any form or format, making this thesis available to interested persons.

L'auteur a accordé une licence irrévocable et non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de sa thèse de quelque manière et sous quelque forme que ce soit pour mettre des exemplaires de cette thèse à la disposition des personnes intéressées.

The author retains ownership of the copyright in his/her thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without his/her permission.

L'auteur conserve la propriété du droit d'auteur qui protège sa thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

ISBN 0-612-18056-5

Canadä

UNIVERSITY OF ALBERTA

LIBRARY RELEASE FORM

NAME OF AUTHOR: **Kerry Woon Sing Ko**

TITLE OF THESIS: **Mechanism and Regulation of Lipid Transfer Protein**

DEGREE: **Doctor of Philosophy**

YEAR THIS DEGREE GRANTED: **1996**

Permission is hereby granted to the University of Alberta Library to reproduce single copies of this thesis and to lend or sell for private, scholarly, or scientific research purposes only.

The author reserves all other publication and other rights in association with the copyright in the thesis, and except as hereinbefore provided, neither the thesis nor any substantial portion thereof may be printed or otherwise reproduced in any material form whatever without the author's prior written permission.

7209 Dow Ave.
Burnaby, B.C.
V5J 3X3
(604) 438-3080

June 24 1996
Date

UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **MECHANISM AND REGULATION OF LIPID TRANSFER PROTEIN** submitted by **KERRY WOON SING KO** in partial fulfillment of the requirements for the degree of **DOCTOR OF PHILOSOPHY** in **MEDICAL SCIENCES** (MEDICINE).

Dr. Shinji Yokoyama (Supervisor)

D. E. Vance

Dr. Dennis E. Vance

Robert O. Ryan

Dr. Robert O. Ryan

Dr. Larry Fliegel

Gordon A. Francis

Dr. Gordon A. Francis

Chris

Dr. Christopher J. Fielding (External Examiner)

May 17th, 1996

Date

ABSTRACT

Mammalian plasma lipid transfer protein (LTP) facilitates the exchange and transfer of lipids between lipoproteins. The mechanism and regulation of neutral lipid transfer between lipoproteins by LTP were investigated by various approaches. A fluorescence assay was developed to monitor transfer of a pyrene-linked neutral lipid analog of cholesteryl ester (CE) between lipid microemulsion particles. LTP activity under these conditions was dependent on the presence of apolipoproteins (apo) such as apoA-I. Activation of LTP was proportional to the degree of saturation of the microemulsion surface by apolipoprotein. LTP isolated from rabbit plasma was found to bind strongly to the microemulsion. Binding of LTP to the microemulsion was not accompanied by drastic change in the secondary structure of LTP. Monoclonal antibodies (mAbs) were raised against rabbit LTP. Three clones selected for study exhibited the following properties: 2-8G was a species-specific, neutralizing mAb which blocked both radioisotopic CE and triacylglycerol (TG) transfer activity of rabbit LTP; 3-9F was a cross-reacting, neutralizing mAb which blocked total radioisotopic neutral lipid transfer activity of either rabbit or human LTP; and 14-8H was a cross-reacting, dissociating mAb since it inhibited radioisotopic TG transfer to much greater extent than CE transfer by rabbit or human LTP. The mechanisms for neutralization versus dissociation of radioisotopic neutral lipid transfer by mAbs were further characterized. Both neutralizing mAbs inhibited binding of LTP to microemulsions, suggesting that they interfere with the interaction between LTP and the lipoprotein surface to achieve inhibition of LTP activity. Dissociating mAb completely blocked transfer of pyrene-neutral lipids between microemulsions or mass transfer of neutral lipids between lipoproteins within incubated human plasma. Furthermore, dissociating mAb did not affect binding of LTP to microemulsions. These results can be interpreted to mean that dissociating mAb partially obstructs the neutral lipid binding site of LTP, affecting radioisotopic TG but not CE transfer, perhaps because of the larger molecular size of TG. Pyrene-CE becomes susceptible to inhibition presumably because of the additional bulk of the pyrene moiety. During incubation of human plasma, dissociating mAb might prevent entry of TG into the neutral lipid binding site of LTP and its subsequent transfer, rendering LTP a CE exchange protein, thus providing evidence for a

heteroexchange mechanism of net neutral lipid mass transfer between lipoproteins by LTP. These results highlight the roles of two structural determinants within the LTP molecule, one involved in lipoprotein binding and the other in neutral lipid binding, both crucial for lipid transfer activity.

ACKNOWLEDGEMENTS

I am thankful to my supervisor, Dr. Shinji Yokoyama, the other members of my supervisory committee, Drs. Dennis Vance and Bob Ryan, and my departmental advisor, Dr. Bill McBlain, for their constant support and encouragement.

I also thank Drs. Cyril Kay and Kim Oikawa of the Department of Biochemistry for their help with circular dichroism spectroscopy, and Drs. Tim Mosmann and Jean-Marie Heslan and Rita Marcotte of the Department of Medical Microbiology and Immunology for helping me prepare my monoclonal antibodies. Drs. Yves Marcel and Ross Milne of the University of Ottawa Heart Institute are thanked for providing TP2 monoclonal antibody.

I am fortunate to have met and worked with many wonderful and talented people during my stay in the Yokoyama lab: technicians Lisa Main and Helena Czarnecka, post-doctoral fellows Drs. Hitoshi Hara, Tom Milner, Akira Komaba, Gordon Francis, Taira Ohnishi, Maki Tsujita, Hidetoshi Kotake, and Jin-Ichi Ito, fellow graduate student Qianqian Li, summer students Vanessa Wong, Alfred Buenaventura, Cedric Tan, Karen Wong, and Samuel Ho, and nutritionists Connie Coffin and Ethel Reteff.

I thank all members of the Lipid Group whom I had the pleasure of overlapping with (September 1989 - May 1996), for sharing good times around the lab, and on the playing fields and rinks at U of A.

Drs. Zemin Yao and Ross Milne, Ms. Sherri Selby, and Mr. Yuwei (Jay) Wang are thanked for reading my thesis.

I am grateful to the Medical Research Council of Canada and the Alberta Heritage Foundation for Medical Research for financial support of my training.

Finally, I wish to express my special appreciation to Sherri Selby and Patrick O'Reilly, for their tremendous friendship, for bailing me out of tight spots on numerous occasions, and for always offering a comfortable refuge from the lab (especially during Hockey Night in Canada) while I was in Edmonton.

TABLE OF CONTENTS

Page

CHAPTER 1

Introduction

1.1 Overview 1

1.2 Mammalian Lipid Transport 2
  1.2.1 Lipoprotein Structure and Classification 2
  1.2.2 Transport of Lipids from the Intestines and Liver 3
  1.2.3 Reverse Cholesterol Transport 5
  1.2.4 Relationship between Lipoproteins and Atherosclerosis 6

1.3 Mammalian Plasma Lipid Transfer 7
  1.3.1 Early Studies 7
  1.3.2 Purification of Lipid Transfer Proteins 8
  1.3.3 Molecular Characterization of LTP 9
  1.3.4 cDNA and Gene for LTP 10
  1.3.5 LTP Levels, Tissue and Cell Expression 11
  1.3.6 Roles of LTP in Lipoprotein Profile and Atherosclerosis 13
  1.3.7 Other Physiological Roles of LTP 15

1.4 Mechanism of LTP Activity 16
  1.4.1 Basic Models 16
  1.4.2 Relationship between CE and TG Transfer Activities of LTP 17
  1.4.3 LTP Distribution in Plasma and Binding to Lipoproteins 18
  1.4.4 Carrying of Neutral Lipids by LTP 19

1.5 Regulation of LTP Activity 19
  1.5.1 Neutral Lipid Specificity of LTP 19
  1.5.2 Modulation of Lipoprotein Composition 20
  1.5.3 Effects of Proteins 21
  1.5.4 Relative Levels of LTP and Lipoproteins 22

1.6 Structure-Function Relationships of LTP 22
  1.6.1 Stability and Protein Folding of LTP 22
  1.6.2 Neutral Lipid Binding Site of LTP 23
  1.6.3 Lipoprotein Binding Site of LTP 24
  1.6.4 Glycosylation of LTP 24
  1.6.5 Alternative Splicing and Mutations of LTP 24

1.7 Scope of Thesis 25

1.8 References 34

CHAPTER 2

Fluorescence Assay for Measuring LTP Activity Used to Study Activation of LTP by Apolipoproteins

2.1 Introduction 58

2.2 Experimental Procedures 59
  2.2.1 Preparation of Lipid Microemulsion Particles 59
  2.2.2 Purification of LTP 59
  2.2.3 Purification of Apolipoproteins 60
  2.2.4 Recording of Fluorescence Spectra 60
  2.2.5 Monitoring of Lipid Transfer 60

2.3 Results 61

2.4 Discussion 63

2.5 References 77

CHAPTER 3

Purification of Rabbit LTP and Characterization of its Interaction with Lipid Microemulsion Particles

3.1 Introduction 82

3.2 Materials and Methods 83
  3.2.1 Materials 83
  3.2.2 Lipoprotein Preparation 84
  3.2.3 Assays of LTP Activity 85
  3.2.4 Purification of Rabbit LTP 86
  3.2.5 Electrophoresis and Immunoblotting 87
  3.2.6 Gel Permeation Chromatography 88
  3.2.7 Protein Sequencing and Amino Acid Analysis 89
  3.2.8 Lipid Microemulsion Particle Binding Analysis 89
  3.2.9 CD Spectroscopy 90
  3.2.10 Other Methods 90

3.3 Results 90
  3.3.1 Purification of Rabbit LTP 90
  3.3.2 Molecular Characterization of Rabbit LTP 91
  3.3.3 Behavior of Rabbit LTP Towards Lipid Microemulsion Particles 92

3.4 Discussion 93

- 3.4.1 Purification of Rabbit LTP 93
- 3.4.2 Molecular Characteristics of Rabbit LTP 94
- 3.4.2 Interaction of Rabbit LTP with Lipid Microemulsion Particles 95

3.5 References 109

CHAPTER 4

Generation of Novel Inhibitory mAbs Against Rabbit LTP and Characterization of Their Different Inhibitory Mechanisms

4.1 Introduction 106

4.2 Experimental Procedures 107
- 4.2.1 Immunizations 107
- 4.2.2 Fusions and mAb Production 108
- 4.2.3 Inhibition Assay Screening and Immunotitration Studies 108
- 4.2.4 Dot Blot Screening 109
- 4.2.5 Pyrene-Neutral Lipid Transfer Assays 110
- 4.2.6 Gel Electrophoresis and Immunoblotting 110
- 4.2.7 Lipid Microemulsion Particle Binding Analysis 110
- 4.2.8 Human Plasma Incubation Studies 110
- 4.2.9 Analytical Methods 112
- 4.2.10 Other Materials 112

4.3 Results 112
- 4.3.1 Raising of mAbs Against Rabbit LTP 112
- 4.3.2 Effects of mAbs on Radioisotopic Neutral Lipid Transfer 113
- 4.3.3 Effects of mAbs on Pyrene-Neutral Lipid Transfer 113
- 4.3.4 Effects of mAbs on LTP Binding to Lipid Microemulsion Particles 114
- 4.3.5 Effects of mAbs on Neutral Lipid Transfer Within Incubated Human Plasma 115
- 4.3.6 Demonstration of Interaction Between mAbs and LTP 118

4.4 Discussion 119
- 4.4.1 Effects of mAbs on Lipid Particle Binding by LTP 119
- 4.4.2 Effects of mAbs on Lipid Transfer Activity of LTP 120

4.5 References 143

CHAPTER 5

Discussion

5.1 General Discussion 147

5.2 Future Directions 150

5.3 References 153

APPENDIX

Derivation of Equation to Calculate the Rate Constant k/L_1 155

LIST OF TABLES

Table		Page
1.1	Composition of major lipoproteins	26
1.2	Chronological list of reports on purification and description of plasma lipid transfer factor(s)	27
3.1	Typical results for purification of rabbit LTP starting from 1.5 L of rabbit plasma	98
3.2	N-terminal amino acid sequencing of purified rabbit LTP	99
3.3	Amino acid analysis of purified rabbit LTP	100
4.1	Effects of mAbs on rate constants for the transfer of pyrene-lipids by rabbit and human LTP between lipid microemulsion particles	126
4.2	Effects of mAbs on changes in lipoprotein lipid composition following inubation of human plasma with LCAT inhibited	127
4.3	Changes in lipoprotein neutral lipid levels on a molar basis in incubated human plasma with LCAT inactivated	128
4.4	Effects of mAbs on changes in lipoprotein lipid composition following incubation of human plasma without LCAT inhibited	129

LIST OF FIGURES

Figure		Page
1.1	Lipoprotein structure and core lipid ratio	29
1.2	Metabolism of hepatic TG-rich lipoproteins	30
1.3	HDL metabolism and the postulated RCT pathway involving LTP	31
1.4	Effects of LTP on lipoprotein lipid composition	32
1.5	Models of neutral lipid binding and transfer by LTP	33
2.1	Elution profile of a lipid microemulsion preparation from a Sepharose CL-4B column	69
2.2	Fluorescence spectrum of lipid microemulsion particles containing pyrene-CE	70
2.3	Relationship between fluorescence ratio of lipid microemulsion particles and microscopic concentration of pyrene-CE within their cores	71
2.4	Changes in fluorescence ratio with time indicating lipid transfer	72
2.5	Effects of LTP and apoA-I on lipid microemulsion particle size	73
2.6	Relationships of rate constants for lipid transfer to LTP concentration	74
2.7	Rate constants of lipid transfer by LTP as a function of saturation of the lipid microemulsion particle surface by apoA-I	75
2.8	Chemical structure of pyrene-CE, or cholesterol 3-(6-(1-pyrene) hexanoate)	76
3.1	Column chromatography during purification of rabbit LTP	101
3.2	SDS-PAGE and Coomassie Blue staining of LTP fractions	103

at different steps of purification

3.3 Immunostaining after SDS-PAGE of LTP fractions at different steps of purification 104

3.4 Superose 12 gel permeation chromatography of purified rabbit LTP 105

3.5 Isoelectric focusing and TP2 immunoblotting of rabbit LTP 106

3.6 Binding of rabbit LTP to lipid microemulsion particles 107

3.7 CD spectra of rabbit LTP taken at 25° C in 10 mM sodium phosphate (pH 7.4), 150 mM NaCl 108

4.1 Immunotitration of CE transfer mediated by rabbit (panel A) and human (panel B) LTP 135

4.2 Immunotitration of TG transfer mediated by rabbit (panel A) and human (panel B) LTP 136

4.3 Effects of mAbs on LTP binding to lipid microemulsion particles 137

4.4 Effects of DTNB or mAb treatment on radioisotopic neutral lipid transfer stimulated by human plasma 138

4.5 Effects of mAbs on radioisotopic CE (panel A) and TG (panel B) transfer between endogenous lipoproteins within incubated human plasma 139

4.6 SDS-PAGE and immunoblotting of LTP 140

4.7 Native-PAGE and immunoblotting of LTP 141

4.8 How mAb 14-8H allows radioisotopic but not net mass transfer of CE 142

5.1 Overall model summarizing the mechanism and regulation of neutral lipid transfer by LTP 152

LIST OF ABBREVIATIONS AND SYMBOLS

Alphabetical list:

a	slope
ACAT	acyl coenzyme A:cholesterol acyltransferase
apo	apolipoprotein
b	y-axis intercept
BCA	bicinchoninic acid
BODIPY	4,4-difluoro-5,7-dimethyl-4-bora-3α,4α-diaza-3-indacene-3-dodecanoate
BPI	bactericidal permeability increasing protein
B_s	binding level at saturation
C	carboxyl
C/EBP	CCAAT/enhancer binding protein
C, c_1, c_2	total, donor, and acceptor pyrene-CE concentrations, respectively; C is also concentration during circular dichroism
CD	circular dichroism
cDNA	complementary deoxyribonucleic acid
CE	cholesteryl ester
CETP	cholesteryl ester transfer protein
CHD	coronary heart disease
cm	centimeter
CM	chylomicron or carboxymethyl
d	density
dL	deciliter
DMEM	Dulbecco's modified Eagle medium
DNA	deoxyribonucleic acid
dpm	disintegrations per minute
DTNB	5,5'-dithiobis-(2-nitrobenzoic acid)
EDTA	ethylenediaminetetraacetic acid
e_1, e_2	donor and acceptor excimeric fluorescence emissions peaks, respectively
E_{470}, E_{395}	emissions fluorescence at 470 and 395 nm, respectively
f	ratio of E_{470}/E_{395}
Fab	fragment antigen binding

FCS	fetal calf serum
FH	familial hypercholesterolemia
FPLC	fast protein liquid chromatography
g	gravity
h	hour
HAT	hypoxanthine-aminopterin-thymidine
HMG-CoA	3-hydroxy-3-methyl glutaryl coenzyme A
HDL	high-density lipoprotein
HPLC	high-performance liquid chromatography
HT	hypoxanthine-thymidine
i.d.	internal diameter
Ig	immunoglobulin
IL	interleukin
k	rate constant
K	thousand
kbp	kilobase pairs
K_d	dissociation constant
kDa	kilodalton
l	pathlength
L	liter
L, L_1, L_2	total, donor, and acceptor core lipid concentration, respectively
LBP	lipopolysaccharide binding protein
LCAT	lecithin:cholesterol acyltransferase
LDL	low-density lipoprotein
LDLR	low-density lipoprotein receptor
log	logarithm
ln	natural logarithm
Lp	lipoprotein
LPDP	lipoprotein-deficient plasma
LPS	lipopolysaccharide
LRP	low-density lipoprotein receptor-related protein
LTC	lipid transfer complex
LTP	lipid transfer protein
M	molar
m_1, m_2	donor and acceptor monomeric fluorescence emissions peaks, respectively

mAb	monoclonal antibody
mg	milligram
min	minute
mL	milliliter
μL	microliter
μM	micromolar
mM	millimolar
mol	mole
mol%	mole percent
MPL	monophosphoryl lipid A
M_r	relative molecular mass
mRNA	messenger ribonucleic acid
n	number of samples
N	amino
nm	nanometer
nM	nanomolar
P	probability value
PAGE	polyacrylamide gel electrophoresis
P_b	bound concentration
PC	phosphatidylcholine
pCMPS	para-chloromercuriphenyl-sulfonate
PEG	polyethylene glycol
pH	acidity
pI	isoelectric point
PL	phospholipid
PMSF	phenylmethylsulfonyl fluoride
P_t	total concentration
PLTP	phospholipid transfer protein
pmol	picomole
PTH	phenylthiohydantoin
q	molar ellipticity
RCT	reverse cholesterol transport
RNA	ribonucleic acid
rpm	revolutions per minute
s	second
SDS	sodium dodecyl sulfate

SEM	standard error of the mean
t	incubation time
TBE	Tris-borate-ethylenediaminetetraacetic acid buffer
TDM	trehalose dimycolate
TG	triglyceride
TNF	tumor necrosis factor
TP2	anti-transfer protein monoclonal antibody 2
U	unit
UV	ultraviolet
V	volt
VLDL	very low-density lipoprotein
V_o	void volume
V_t	total volume
v/v	volume per total volume
w/v	weight per total volume
w/w	weight per weight

Amino acids are referred to by their 3-letter abbreviations

Non alphabetic symbols:

°C	degrees Celsius
%	percent

CHAPTER 1

Introduction

1.1 Overview

Lipids, being hydrophobic and poorly soluble in aqueous environments, are transported within the mammalian circulatory system by incorporation into lipoproteins. The mammalian lipid transport system supplies glycerolipids from the intestines and liver to peripheral tissues such as muscle and adipose where the fatty acid component is metabolized for energy production or storage, respectively. Another important role of this system is delivery of excess cholesterol from peripheral tissues back to the liver, the only location in the body where it can be catabolized, for elimination from the body, termed reverse cholesterol transport (RCT). To meet these roles, the mammalian lipid transport system employs numerous complex biochemical processes for the assembly and secretion, modification while in circulation, and receptor-mediated clearance of lipoproteins. Disturbances of this system as a result of primary and secondary dyslipidemias (reviewed in Francis, 1995) are often associated with premature atherosclerosis and coronary heart disease (CHD).

Processes which modify the lipid composition of lipoproteins in the circulation serve important roles within the mammalian lipid transport system. Lipases release fatty acids from the phospholipid (PL) and triglyceride (TG) components of lipoproteins for uptake by tissues. Lecithin:cholesterol acyltransferase (LCAT) esterifies free cholesterol on the surface of lipoproteins and is important in the formation of high-density lipoprotein (HDL). Most of the lipid components of lipoproteins have low capacity for spontaneous movement between separate lipoproteins. However, protein mediated transfer of lipids between circulating lipoproteins occurs, with plasma lipid transfer protein (LTP) being responsible for transfer of cholesteryl ester (CE) and TG, as well as there being a separate phospholipid transfer protein (PLTP) involved in PL transfer. LTP activity is important in RCT, as well as influencing the lipoprotein profile, leading to important implications regarding atherosclerosis.

This thesis describes studies directed to further characterize the mechanism and regulation of the neutral lipid transfer activity of LTP. Human and rabbit plasma provided sources of LTP activity for our studies and development of artificial lipid microemulsion particles and monoclonal antibodies (mAbs) against LTP provided novel tools for pursuing various questions about the mechanism and regulation of LTP activity.

The remainder of this chapter provides a brief summary of the mammalian lipid transport system as well as reviewing the background and physiology of plasma lipid transfer activity. This is followed by a summary of the major findings from various biochemical approaches to investigate the mechanism and regulation of LTP activity. A final section on structure-function relationships of LTP summarizes contemporary findings using recombinant DNA techniques to approach these same issues.

1.2 Mammalian Lipid Transport System

1.2.1 Lipoprotein Structure and Classification

Lipoproteins are spherical microemulsion particles consisting of a core of non-polar, neutral lipids (CE and TG) surrounded by a monolayer of amphipathic lipids (PL and free cholesterol) as well as associated apolipoproteins (Shen *et al.*, 1977). Small amounts of neutral lipids distribute into the surface monolayer according to phase behavior, and some cholesterol distributes to the cores of lipoproteins with increasing particle size. The major apolipoproteins are synthesized mainly in the liver and intestines, and can be divided into 3 categories (Pownall and Gotto Jr., 1992). Group 1 apolipoproteins include apoA-I, A-II, A-IV, C-I, C-II, C-III, and E. In general, these are small, exchangeable, and possess repeated regions of amphiphilic, alpha-helical structure which may be involved in lipid binding. Group 2 apolipoproteins include apo(a) and apoB. Both of these proteins are large and non-exchangeable. ApoB is an extremely hydrophobic protein, with its lipophilicity determined in part by regions of hydrophobic residues and amphipathic beta structure. Apo(a) is highly polymorphic, and possesses multiple characteristic kringle structures. Group 3 apolipoproteins include apoD, LTP and PLTP. These are generally small, exchangeable, and can bind and carry lipid.

The plasma lipoproteins are heterogeneous in size, lipid and protein composition, permitting them to be separated into 4 major density classes by ultracentrifugation: chylomicron (CM), very low-density lipoprotein (VLDL), low-density lipoprotein (LDL), and HDL (subclasses HDL_2 and HDL_3 in humans). These correspond to origin, pre-beta, beta, and alpha migrating bands obtained by agarose electrophoresis, which separates lipoproteins according to charge. Immunochemical techniques also permit separation or detection of lipoprotein fractions according to apolipoprotein composition and precipitation methods exist to separate apoB-containing lipoproteins from non-apoB-containing ones. All these methods of lipoprotein separation and analysis have been summarized together (Mackness and Durrington, 1992).

The 4 classes of lipoproteins classified on the basis of their flotation density also range in size, core ratio of TG to CE, and lipid to protein ratio, with CM having highest values for all these aspects, which all decrease with increasing lipoprotein density (Fig. 1.1 and Table 1.1)

1.2.2 Transport of Lipids from the Intestines and Liver

After a meal, the products of fat digestion (fatty acids, monoglycerides, lysoPC, and cholesterol) enter the enterocytes from mixed micelles in the intestinal lumen and undergo esterification reactions within the smooth endoplasmic reticulum. Long chain fatty acids (> 14 carbons) undergo esterification with monoacylglycerides to form TG and with lysoPC to form PC. The enzyme acyl coenzymeA:cholesterol acyltransferase (ACAT) esterifies cholesterol to CE, mainly cholesteryl oleate (Miller, 1990). These lipids are then used to assemble chylomicron (CM) particles, which are secreted into the lymph and enter the venous circulation at the thoracic duct.

CM are the largest, least dense lipoproteins with cores that are rich in TG. The size and lipid composition of CM are regulated by diet and lipid availability (Davis, 1991). Human CM particles contain a single copy of a truncated version of apoB consisting of the N-terminal 48% of the full length protein, termed apoB-48, which arises from a novel RNA editing process occurring in the intestines. Newly secreted CM receive C and E apolipoproteins from HDL and travel within the circulation to tissues with high TG demands, such as muscle, adipose tissue, and lactating mammary gland. There, in a reaction requiring apoC-II, lipoprotein lipase bound to glycosaminoglycans on the capillary endothelial cell surface hydrolyzes the TG component (Dolphin, 1992), liberating fatty acids which can be taken up by parenchymal cells or bound to albumin and transported to other tissues, mainly liver. After depletion of much of their core lipids, CM remnants are released from the capillaries and rapidly removed from the circulation, with liver being the major clearance site. The candidate receptors for this clearance are the low-density lipoprotein receptor (LDLR) and low-density lipoprotein receptor-related protein (LRP), through recognition of ligands other than apoB-48, which lacks the receptor-binding domain for recognition by the LDLR. Efficient clearance of CM remnants by the liver seems to involve interactions with cell surface proteoglycans and various ligands, which may form a bridge between the remnant particle and LRP or the proteoglycans, including lipoprotein lipase, hepatic lipase, and apoE (Herz and Willnow, 1995). Thus, in man, CM clearance is rapid and they should not contribute much to plasma TG levels during the postabsorptive state.

The liver produces lipids by *de novo* synthesis from glucose and fatty acids as well as re-using intermediates produced from the uptake and catabolism of remnant lipoproteins, for the assembly and secretion of VLDL. VLDL are also large and TG rich, being the major TG carrying lipoprotein in the plasma. In humans, liver ACAT activity is low so much of their VLDL CE content arises from cholesterol esterification within the plasma on HDL by LCAT, followed by transfer to VLDL involving a lipid transfer protein. ApoB and lipid synthesis are essential for VLDL assembly and secretion (Davis, 1991).

VLDL enter the venous circulation from the hepatic vein, where they also acquire C and E apolipoproteins and travel to peripheral tissues before undergoing lipolytic processes, similar to CM. VLDL remnants released from the capillaries are also removed by the liver by processes relying on apoE, similar to CM remnant clearance. However, in humans, about one-third of VLDL remnants undergo lipolysis by hepatic lipase, leading to further reduction of the core TG content and shedding of exchangeable apolipoproteins. This results in formation of LDL, which contains only one copy of apoB (Kane and Havel, 1989) and is CE rich, carrying about two-thirds of the total cholesterol in human plasma.

LDL are cleared from circulation mainly through recognition of apoB by the liver LDL receptor (LDLR) (Dietschy *et al.*, 1993). Human mutations resulting in defects in LDLR activity cause familial hypercholesterolemia (FH), providing a clear link between elevated plasma cholesterol levels and CHD (Brown and Goldstein, 1986). LDL are small enough to pass the endothelial barrier and enter the extravascular compartment, to supply cholesterol to peripheral tissues with high cholesterol demands, such as the adrenal gland, gonads and skin. Other mechanisms for removal of LDL include uptake by macrophage scavenger receptors, recognizing modified LDL, which may be involved in foam cell formation and development of atherosclerosis (Krieger, 1992). The plasma metabolism of TG-rich lipoproteins, using hepatic VLDL as an example, is depicted in Fig. 1.2.

1.2.3 Reverse Cholesterol Transport

HDL are the smallest lipoproteins and the other major cholesterol carrier in human plasma. The major apolipoproteins of HDL, apoA-I and A-II, are synthesized by the liver and intestines. Their assembly process is unclear and HDL apolipoproteins may be secreted lipid-free or as discoidal particles containing PL and free cholesterol which form spherical particles through LCAT action in the plasma. However, direct evidence of intracellular formation of discoidal HDL within the secretory pathway is lacking. Extracellular HDL assembly would involve extraction of PL and cholesterol from plasma membranes or lipoproteins to form discoidal HDL, followed by LCAT activity to form spherical particles (Hara and Yokoyama, 1991). Alternatively, secreted HDL apolipoproteins may associate with existing lipoproteins, and become transferred to HDL during lipolysis. Spherical HDL undergo a cycle of enlargement due to LCAT activity and exchange of CE for TG mediated by LTP, followed by lipolysis by hepatic lipase, leading to interconversion between HDL_2 and HDL_3 (Morton, 1990).

Demonstration of the plasma cholesterol esterification reaction by LCAT lead to the proposal for its involvement in transport of excess peripheral tissue cholesterol to the liver for catabolism, a process termed RCT (Glomset, 1968). Additional steps of this pathway have been provided through numerous subsequent studies. Removal of free cholesterol by HDL from peripheral cells involves desorption of cholesterol through the aqueous phase (reviewed in Johnson *et al.*, 1991) and possibly binding of HDL to proteins in the plasma membrane along with protein kinase C-mediated signal transduction (reviewed in Pieters *et al.*, 1994). The initial cholesterol acceptor seems to be a small HDL subfraction which is lipid-poor and has prebeta electrophoretic mobility (Fielding and Fielding, 1995). Such particles are good substrates for LCAT, which esterifies the free cholesterol, predominantly to cholesteryl linoleate (Miller, 1990), requiring apoA-I as a co-factor. Then, several routes are possible for the return of the removed cholesterol to the liver. Large HDL can acquire apoE and be removed by LDLR and LRP. In a process known as selective uptake, HDL CE can be taken up by cells without particle uptake (Rinninger *et al.*, 1994; Acton *et al.*, 1996). However, in species possessing plasma LTP activity, such as humans, the major route is transfer of CE from HDL to TG-rich lipoproteins in exchange for TG, with CE reaching the liver during clearance of lipoprotein remnants (McPherson, 1993). HDL catabolism is complex because the lipid and apolipoprotein components are both subject to transfer to other lipoproteins and the only particle uptake mechanism is through recognition of apoE. A major site for turnover of free HDL apolipoprotein is the kidney (Horowitz *et al.*, 1993). Plasma metabolism of HDL and the pathway for RCT involving LTP are summarized by illustration in Fig. 1.3.

Following its return to the liver, cholesterol is degraded to bile acids as well as secreted directly in the bile. Much of the secreted bile acids and some of the cholesterol are recovered through the entero-hepatic circulation, with the remainder lost in the feces. The net cholesterol lost from the body must be balanced by dietary intake and endogenous synthesis, which appears to occur primarily in extrahepatic tissues (Dietschy *et al.*, 1993).

1.2.4 Relationship between Lipoproteins and Atherosclerosis

Atherosclerosis is responsible for 50% of all mortality in industrialized nations. Epidemiological evidence indicates elevated levels of serum cholesterol as one of the major risk factors for developing premature CHD (Gotto Jr *et al.*, 1990). Genetic forms of hyperlipidemia in man and animals have been identified which produce CHD in absence of other risk factors, such as FH (Brown *et al.*, 1981). Clinical trial results have shown that lowering cholesterol levels is effective in prevention and regression of atherosclerosis (Brown *et al.*, 1990). Serum cholesterol levels are strongly correlated with LDL cholesterol levels, which may be the major culprit in atherosclerotic plaque formation. On the other hand elevated HDL cholesterol levels may be protective against development of CHD (Gordon and Rifkind, 1989).

The response to injury hypothesis is a major model for the pathogenesis of atherosclerosis (Ross, 1993). Within this hypothesis, insults to the arterial wall lead to an excessive inflammatory-fibroproliferative response. Oxidative modification of LDL provides an explanation for its atherogenicity in hypercholesterolemia (Steinberg *et al.*, 1989). Oxidized LDL can play a role in endothelial injury by being cytotoxic and stimulating migration of monocytes and lymphocytes into the arterial intima. Furthermore, oxidation of LDL produces a form recognized by monocyte-derived macrophage scavenger receptors, leading to foam cell and fatty streak formation. Alternative hypotheses for the pathogenesis of atherosclerosis include response-to-retention, citing retention of LDL within the atery wall as the key event in early atherogenesis (Williams and Tabas, 1995)

The anti-atherogenic properties of HDL may relate to their role in RCT. HDL can remove cholesterol from CE-loaded foam cells in culture and infusion of HDL produces regression of atherosclerosis in rabbits. While low HDL cholesterol levels in LCAT deficiency and Tangier disease are not associated with atherosclerosis, apoA-I deficiency prediposes subjects to premature CHD (Ng *et al.*, 1994). Secondly, HDL may protect LDL from oxidation (Parthasarathy *et al.*, 1990; Kunitake *et al.*, 1992) or aggregation (Khoo *et al.*, 1990) to prevent its uptake by macrophages. Thirdly, diminished HDL cholesterol levels may be secondary to abnormal lipoprotein remnant metabolism resulting from overproduction, or inefficient lipolysis or clearance, leading to accelerated and prolonged CE transfer to VLDL and CM mediated by LTP (reviewed in Tall, 1990).

1.3 Mammalian Plasma Lipid Transfer

1.3.1 Early Studies

Nichols and Smith (1965) described mass transfer of CE and TG between lipoprotein classes following incubation of human plasma. Akanuma and Glomset (1968) also noted that CE of VLDL became labeled when human plasma was incubated with isotopic free cholesterol, although plasma cholesterol esterification occurred mainly on HDL. It was also apparent that the source of VLDL CE was markedly different in man and rat, with rat VLDL being abundant in CE molecular species reflecting liver ACAT synthesis while human VLDL CE composition reflected species generated by

LCAT activity (reviewed in Glomset, 1968). Several years passed following these initial indications of neutral lipid transfer between lipoproteins in humans before interest in the details of this process resumed.

Lipoprotein-free plasma from rabbit (Zilversmit *et al.*, 1975) and man (Sniderman *et al.*, 1978, Barter and Jones, 1979) were shown to facilitate radioisotopic CE transfer *in vitro* between certain isolated lipoprotein classes, such as LDL and HDL, while plasma of certain species, such as rat, lacked CE transfer activity (Barter and Lally, 1978). No net mass transfer occurs in this instance, so radiolabel movement is indicative of exchange. Bidirectionality of radioisotopic transfer of CE (Barter and Lally, 1979) and TG (Barter *et al.*, 1979) between all lipoprotein pairs was demonstrated. Net mass transfer of neutral lipids between some lipoprotein pairs, such as VLDL and HDL, was also shown to be stimulated by plasma (Hopkins *et al.*, 1980; Marcel *et al.*, 1980), as well as *in vivo* transfer of radioisotopic CE from HDL to other lipoproteins in man (Nestel *et al.*, 1979) and rabbit (Ha *et al.*, 1981).

1.3.2 Purification of Lipid Transfer Proteins

Between 1978 and 1987, there are at least 12 reports on purification of the protein(s) responsible for plasma CE and/or TG transfer activity (Table 1.2). Assays measuring radioisotopic lipid transfer between isolated lipoproteins were usually employed to monitor the degree of purification. The results from most of these papers described obtaining of a hydrophobic glycoprotein having M_r of approximately 65,000 found in human and rabbit plasma, with an acidic pI, and which was resistant to inactivation by high temperature and distinct from LCAT. Sometimes, TG or PC transfer was monitored as well as CE transfer activity; Morton and Zilversmit (1982) also monitored retinyl ester and cholesteryl ether transfer. An unusual report by Busch *et al.* (1987) described isolation of a low M_r catalytic domain as well as a heparin binding domain, while the transfer protein studied by Chajek and Fielding (1978) had characteristics similar to apoD. Subsequently, CE transfer activity and apoD were clearly resolved (Morton and Zilversmit, 1981a). However, none of these reports provided sufficient biochemical data to establish the identity or purity of the proteins obtained by the various groups. The question of whether a single protein or separate proteins were responsible for CE, TG, and PL transfer also remained unanswered.

In 1987, two groups (Hesler *et al.*, 1987; Jarnagin *et al.*, 1987) described purification of very similar M_r 74,000 proteins having CE and TG transfer activity (Table 1.2). Amino acid composition and N-terminal protein sequence data in both reports showed strong resemblance, confirming the identity and homogeneity of their separately isolated transfer proteins. Many characteristics of this protein, called LTP herein, otherwise referred to as cholesteryl ester transfer protein (CETP) or LTP-I in the literature, were similar to many of the proteins described earlier in Table 1.2, such as molecular weight, hydrophobicity, pI, and ability to facilitate TG as well as CE transfer. Thus, at least one human plasma protein could be isolated with CE transfer activity. This protein also possessed TG transfer activity, but at this time, there was also the possibility of additional proteins possessing TG transfer activity alone (Table 1.2; Rajaram *et al.*, 1980) or additional CE transfer proteins.

Several groups monitored PC transfer activity during purification of proteins with CE transfer activity (Table 1.2); Harmony's group (Ihm *et al.*, 1980; 1982a) found that human transfer protein could catalyze PC as well as CE transfer while transfer protein purified the same way from rat plasma only transferred PC. Tall *et al.* (1983) showed that CE transfer activity was associated with PL exchange activity, and could be chromatographically resolved from PL mass transfer activity in human plasma. Finally, removal of activity by mAb demonstrated conclusively that LTP was responsible for all radioisotopic CE and TG transfer activity in human plasma, as well as a portion of PL transfer activity (Hesler *et al.*, 1988). Alber's group (Tollefson *et al.*, 1988a) was the first to isolate a human plasma lipid transfer protein specific for PC, called PLTP herein as well as by others, or alternatively LTP-II. Jauhiainen *et al.* (1993) and Tu *et al.* (1993) have also apparently purified this protein (Table 1.2).

1.3.3 Molecular Characterization of LTP

LTP purified from human plasma consists of a single polypeptide and its high content (45%) of non-polar amino acid residues make it more hydrophobic than all known apolipoproteins, including apoB (Hesler *et al.*, 1987). LTP secreted from HepG2 cells has an M_r of around 74,000 and contains N-linked carbohydrates and sialic acids while intracellular LTP has an M_r of 58,000 (Swenson *et al.*, 1987). Isoelectric focusing of LTP reveals a slightly acidic pI and presence of 2 isoforms (Hesler *et al.*, 1987). One study showed LTP to consist of multiple pI isoforms which could be condensed into two bands by isoelectric focusing, with pI's of approximately 5.6, after neuraminidase treatment (Kato *et al.*, 1989). LTP behaves as a monomer during gel permeation chromatography (Hesler *et al.*, 1987; Swenson *et al.*, 1988) and sedimentation equilibrium ultracentrifugation (Ohnishi *et al.* 1994a). The functional unit of rabbit LTP determined by radiation inactivation is the monomer (Ierides *et al.*, 1985). Self association of LTP into dimers and multimers has been observed by native gradient gel electrophoresis (Hesler *et al.*, 1987; Swenson *et al.*, 1989) or quasielastic light scattering (Takahashi *et al.*, 1993). LTP displays saturable binding to 26 nm lipid microemulsion particles prepared from sonicated PC and TG with a K_d of 12 nM, which is at least 10-times lower than for binding of exchangeable apolipoproteins to the same particles (Ohnishi *et al*, 1994a). This study also found that a solution of LTP forming a monolayer at the air-water interface shows extremely high collapse pressure, another indication of high affinity for hydrophobic surfaces. LTP binds to PL monolayers with an affinity equivalent to that of apolipoproteins, but with a low equilibrium surface pressure of LTP compared to the apolipoproteins, suggesting that LTP may not penetrate the lipid surface deeply (Weinberg *et al.*, 1994). The exclusion pressure of LTP from PL monolayers is similar to the pressure of the HDL surface, consistent with LTP being able to adsorb and desorb from the lipoprotein surface.

1.3.4 cDNA and Gene for LTP

The cDNA sequence of human LTP codes for a polypeptide of 476 amino acid residues, with a molecular mass of 53,108 daltons (Drayna *et al.*, 1987a). This predicted sequence is extremely hydrophobic and contains 4 potential Asn-linked glycosylation sites. There is greater than 20% overall amino acid sequence homology between LTP and two other proteins, mammalian lipopolysaccharide binding protein (LBP) and bactericidal permeability increasing protein (BPI), indicating that LTP arose from an ancient family of proteins which bind lipopolysaccharide (LPS) (reviewed in Tall, 1993). PLTP turns out to be another member of this family, sharing 20% protein sequence homology with LTP (Day *et al.*, 1994). The cDNA for LTP from rabbit (Nagashima *et al.*, 1988), monkey (Pape *et al.*, 1991a), and partially from hamster (Jiang *et al.*, 1991) are highly similar, sharing greater than 81% sequence homology with human LTP cDNA at the nucleotide and amino acid levels. An alternatively spliced form of LTP cDNA, missing exon 9, is expressed in all human tissues and produces inactive, poorly secreted protein (Inazu *et al.*, 1992) which may regulate LTP secretion (discussed below).

The single gene for human LTP is located on the long arm of chromosome 16 (Lusis *et al.*, 1987), near the gene for LCAT, and is approximately 25 kbp comprised of 16 exons (Agellon *et al.*, 1990). The intron/exon organization is conserved between LTP and PLTP (Tall, 1995) while PLTP is located on chromosome 20, along with other members of the LPS binding protein family (Day *et al.*, 1994). While the LTP cDNA coding region did not show any major homology with other proteins of mammalian lipid transport other than PLTP, there is a pentapeptide within the signal sequence common to lipoprotein lipase, apoA-I, and apoA-IV which may have a conserved function related to translational or post-translational regulation of expression (Agellon *et al.*, 1990).

Two point mutations within intron 14 of the LTP gene produce defective splicing of mRNA and LTP is undetectable in plasma of individuals homozygous for these null alleles (Brown *et al.*, 1989; Inazu *et al.*, 1994). A missense mutation (Inazu *et al.*, 1994) and nonsense mutation (Gotoda *et al.*, 1993) have also been identified in Japanese populations, as well as several novel missense and deletion mutations in German populations (reviewed in Tall, 1995). Restriction fragment length polymorphisms in DNA at the LTP locus have been described (Drayna *et al.*, 1987b; Freeman *et al.*, 1990) and LTP was shown to have "level gene" effects on HDL levels (Kondo *et al.*, 1989), as well as "variability gene" effects on LDL and total cholesterol levels (Berg *et al.*, 1989).

1.3.5 LTP Levels, Tissue and Cell Expression

Plasma LTP activity is species-specific among vertebrates, with humans having intermediate levels and rabbits high levels, while many other common lab animals such as dogs, rats, and mice (Jiao *et al.*, 1990) have low levels (Ha and Barter, 1982). Among primates, the concentration of LTP in human plasma is approximately 1.7 mg/L, with women having slightly higher levels (Marcel *et al.*, 1990) while monkeys have more than 10 times this amount (Quinet *et al.*, 1991). Alcohol lowers human plasma LTP activity

(Hirano *et al.*, 1992) and concentration (Savolainen, *et al.*, 1990; Hannuksela *et al.*, 1992). Exercise also lowers LTP concentrations (Seip *et al.*, 1993) and marathon runners have lower (Serrat-Serrat *et al.*, 1993) while obese people (Dullaart *et al.*, 1994a) and smokers (Dullaart *et al*, 1994b) have higher activity levels.

Plasma LTP concentration is increased in hypercholesterolemia and some hyperlipidemias (Tall *et al.*, 1987; Bagdade *et al.*, 1991a; McPherson *et al.*, 1991; Tall, 1993). In nephrotic syndrome, LTP concentration (Moulin *et al.*, 1992) and activity (Subbaiah and Rodby, 1994) are increased. There is also increased LTP activity in insulin-dependent (Dullaart *et al.*, 1989; Bagdade *et al.*, 1991b) and noninsulin-dependent (Bagdade *et al.*, 1993; Kahri *et al.*, 1994) diabetes mellitus. LTP activity is also higher in humans during pregnancy (reviewed in Tall, 1995). On the other hand, the hyperlipidemia of hypothyroidism is accompanied by decreased LTP activity (Dullaart *et al.*, 1990). Corticosteroid treatment normalizes the LTP concentration of nephrotic syndrome (Moulin *et al.*, 1992). Probucol treatment raises LTP activity (Franceschini *et al.*, 1989; Chiesa *et al.*, 1993) and concentration (McPherson *et al.*, 1991a). Treatments with HMG-CoA reductase inhibitors do not affect LTP activity (Bagdade *et al.*, 1990) or concentration (Cheung *et al.*, 1993; Guerin *et al.*, 1995) while decreased activity was found in one report (Ahnadi *et al.*, 1993). Fish oil treatment was also found to lower plasma LTP activity (Abbey *et al.*, 1990) and concentration (Bagdade *et al.*, 1992).

LTP mRNA is highly expressed in mammalian liver (Drayna *et al.*, 1987a; Nagashima *et al.*, 1988, Quinet *et al.*, 1991) and adipose (Jiang *et al.*, 1991, Quinet *et al.*, 1991). A high cholesterol, high fat diet increases LTP activity in plasma of rabbits (Son and Zilversmit, 1986) and monkeys (Pape *et al.*, 1991a; Quinet *et al.*, 1991) with increased LTP mRNA in the liver (Quinet *et al.*, 1990) and liver and adipose (Pape *et al.*, 1991a; Quinet *et al.*, 1991), respectively. Cholesterol feeding leads to increased plasma LTP activity (Stein *et al.*, 1990; Quig *et al.*, 1991) and adipose mRNA (Jiang *et al.*, 1991) in hamster, increased activity and liver mRNA in rabbit (Warren *et al.*, 1991) and increased plasma activity in man (Martin *et al.*, 1993). Probucol treatment leads to an increase in hamster, but decrease in human, adipose LTP mRNA level (Quinet *et al.*, 1993a).

Various cell types including HepG2 (Faust *et al.*, 1989; Swenson *et al.*, 1987), CaCo-2 (Faust and Albers, 1988) and monocyte-derived macrophages

(Faust *et al.*, 1990) secrete LTP. However, the abundance of mRNA is low in transformed and long-term cultured primary cells; furthermore, cholesterol loading of some cultured cells increases LTP secretion (reviewed in Tall, 1993). Liver LTP mRNA expression has been attributed to both parenchymal (Agellon *et al.*, 1992) and non-parenchymal (Pape *et al.*, 1991b) cells, and there are species variations in the major cell source of mRNA (Tall, 1993). The transcription factor C/EBP transactivates the LTP gene and low levels of C/EBP in transformed and long-term cultured cells may account for low LTP mRNA in these cells (Agellon *et al.*, 1992). Butyrate treatment, possibly acting as a differentiation agent, also induces expression of LTP mRNA in HepG2 cells (Sperker *et al.*, 1993). The natural flanking regions of the human LTP gene confers transcriptional regulation by dietary cholesterol (Jiang *et al.*, 1992) and corticosteroid treatment (Masucci-Magoulas *et al.*, 1995) to an LTP transgene expressed in mice.

1.3.6 Roles of LTP in Lipoprotein Profile and Atherosclerosis

Compared to humans, rats have efficient CM and VLDL clearance and lack LTP, resulting in plasma cholesterol accumulation mainly in HDL (Oschry and Eisenberg, 1982). Injection of exogenous LTP into rats leads to reduction of CE levels and size of their large, apoE-rich HDL and increase of CE in LDL and VLDL (Ha *et al.*, 1985; Quig *et al.*, 1986; Groener *et al.*, 1989). Disappearance of injected LTP from rat plasma is biphasic, with 70% loss within 6 h (Quig *et al.*, 1986). Conversely, blocking LTP by injection of antibody into rabbits leads to increase in HDL CE and particle size (Abbey *et al.*, 1989) without an increase in particle number (Whitlock *et al.*, 1989). Similarly, inhibition of LTP in incubated human plasma lead to increased CE in LDL and HDL, substrates of LCAT (Yen *et al.*, 1989).

Increases in plasma LTP concentration are negatively correlated with HDL cholesterol levels in man (Groener *et al.*, 1984) and monkeys (Quinet *et al.*, 1991), and positively correlated with (VLDL+LDL) and LDL cholesterol levels, respectively. It was also shown in man that LTP concentrations were positively correlated with VLDL cholesterol levels in hyperlipoproteinemia (McPherson *et al.*, 1991b). Null and missense alleles of LTP are prevalent determinants of decreased plasma LTP concentration or activity and increased HDL cholesterol levels in the Japanese population (Inazu *et al.*, 1990; Yamashita *et al.*, 1991; Inazu *et al.*, 1994), with LTP deficiency leading to major increases in concentration, size (Duverger *et al.*, 1995), and apoE content (Yamashita *et al.*, 1990) of apoA-I-containing lipoproteins, as well as enrichment of LDL with TG (Bisgaier *et al.*, 1991). Moreover, HDL from LTP deficient subjects is poorer in promoting cholesterol efflux, and their plasma cholesterol esterification rate is lower than normal controls (Ohta *et al.*, 1995).

Transgenic expression of LTP in mice, which lack LTP, leads to reduction of HDL cholesterol levels (Agellon *et al.*, 1991), and apoA-I levels and HDL size (Marotti *et al.*, 1992). The reduction in HDL cholesterol was greater if the mice co-expressed human apoA-I, demonstrating a positive interaction between LTP and human apoA-I, or elevated levels of human-like HDL thus formed (Hayek *et al.*, 1992). Transgenic co-expression of human apoA-I, and apoC-III produce a lipoprotein profile of polydisperse HDL and hypertriglyceridemia and introduction of the LTP transgene into this background lead to markedly decreased HDL size by increased CE and apoA-I catabolism (Hayek *et al.*, 1993). There is increased apoA-I turnover in LTP transgenic mice when LTP promotes TG transfer to HDL, followed by lipolysis with apoA-I shedding (Melchior *et al.*, 1994), and conversely, apoA-I turnover is decreased in human LTP deficiency (Ikewaki *et al.*, 1993). Introduction of the apoA-II transgene into the LTP/apoA-I/apoC-III trangenic mice lead to increased CE in VLDL with accumulation of TG in HDL, indicating that apoA-II didn't inhibit LTP but prevented HDL remodeling by hepatic lipase (Zhong *et al.*, 1994). Transgenic co-expression of LTP and receptor-binding defective apoE in mice also showed that LTP increased CE of beta-VLDL without influencing hyperlipidemia, which was mainly due to the ratio of defective to normal apoE in VLDL, in this model of Type III hyperlipoproteinemia (Fazio *et al.*, 1994).

Transgenic expression of very high levels of LTP in mice was reported to produce severe atherosclerosis (Marotti *et al.*, 1993) and fatty liver (Blake *et al.*, 1994). The effect of a high-fat, high cholesterol diet on generating a atherogenic lipoprotein profile is also enhanced in LTP transgenic mice (Marotti *et al.*, 1992). Also, the extent of coronary artery atherosclerosis is positively correlated with plasma LTP concentration in monkeys fed a high-fat, high cholesterol diet (Quinet *et al.*, 1991). Although only anecdotal at present, human LTP deficiency is associated with longevity and freedom from atherosclerosis (reviewed in Tall, 1995). Findings of increased LTP concentration or activity in some primary and secondary hyperlipidemias associated with accelerated atherosclerosis, and of reductions with exercise or alcohol intake (discussed above), and that species with low LTP activity are resistant to dietary atherosclerosis (Tall, 1986) also tend to suggest that elevations in LTP activity are pro-atherogenic.

However, mice made hypertriglyceridemic by transgenic expression of human apoA-I and C-III have less severe atherosclerosis when the LTP transgene is introduced (Hayek *et al.*, 1995). Transgenic expression of LTP in mice also results in increased VLDL and LDL cholesterol and plasma apoB levels, resulting from down-regulation of hepatic LDL receptors, presumably by increased RCT (Jiang *et al.*, 1993), and conversely, LDL catabolic rate is increased in genetic LTP deficiency (Ikewaki *et al.*, 1995). Although genetic LTP deficiency raises HDL cholesterol levels, affected individuals are being found with premature coronary heart disease (Inazu *et al.*, 1994) and the prevalence of mutations in the Japanese population should allow thorough studies of the relationship between LTP deficiency and atherosclerosis. Furthermore, probucol treatment as antiatherogenic therapy lowers LDL and HDL cholesterol levels while increasing plasma LTP levels (discussed above). Thus, there is also evidence that LTP can significantly contribute to RCT, as well as uncertainty whether the increased HDL levels afforded by LTP deficiency is really beneficial against atherosclerosis.

Probably, in humans, the benefits of having the route for RCT afforded by LTP outweigh the drawbacks of the somewhat reduced HDL cholesterol levels accompanying LTP activity, when TG-rich lipoprotein remnant clearance is normal. However, when remnant clearance is slow, for instance due to defects in lipolysis or receptor function, LTP activity may exacerbate accumulation of atherogenic apoB containing lipoproteins.

1.3.7 Other Physiological Roles of LTP

Lipoprotein-deficient plasma stimulates the transfer of CE from PL bilayers to HDL (Young and Brecher, 1981), suggesting a role for LTP in lipid transfer from biological membranes to lipoproteins. Lipoprotein-depleted plasma or partially pure LTP were also able to stimulate radioisotopic efflux of extracellular and cell-surface associated CE, simulating an aortic deposit, into the incubation medium (Stein *et al.*, 1985; Stein *et al.*, 1986). LTP apparently stimulated selective uptake of radioisotopic CE from HDL into HepG2 and certain other cells (Granot *et al.*, 1987), although this was subsequently shown to involve transfer to secreted lipoproteins and cell uptake (Rinninger and Pittman, 1989). LTP was shown to stimulate radioisotopic and net efflux of CE and TG from macrophages into the incubation medium, which could be enhanced by presence of lipoproteins (Morton, 1988a), although removal from reticuloendothelial cell *in vivo* did not occur (Stein *et al.*, 1991). The induction of peripheral tissue LTP mRNA by cholesterol feeding (Jiang *et al.*, 1991) may indicate a role of LTP in lipid transport within the interstitial fluid of the extravascular compartment.

Incubation of HDL_2 with LTP in the presence of TG-rich lipoproteins lead to formation of subpopulations of smaller HDL (Hopkins *et al.*, 1985, Ellsworth *et al.*, 1987), which was enhanced by inclusion of lipases (Deckelbaum *et al.*, 1986; Newnham and Barter, 1990) or unesterified fatty acids (Barter *et al.*, 1990a). Shedding of apoA-I from HDL when incubated with VLDL, LTP and hepatic lipase leads to formation of discoidal HDL (Clay *et al.*, 1992), and movement of CE from HDL by LTP can generate pre-beta HDL (Kunitake *et al.*, 1992). Conversion of HDL into smaller (and larger) subpopulations can also be mediated by LTP in the absence of lipid transfer to another lipoprotein class (Ha *et al.*, 1983; Lagrost *et al.*, 1990), which was also enhanced by free fatty acids (Lagrost and Barter, 1991). The conversion factor purified from human plasma, although not identified as LTP, was activated by apoA-IV (Barter *et al.*, 1988) whereas apoA-II was found to inhibit LTP conversion of reconstituted HDL (Rye *et al.*, 1992). More recently, PLTP has been demonstrated to have HDL conversion activity (Jauhianen *et al.*, 1993; Tu *et al.*, 1993; Albers *et al.*, 1995), indicating that both LTP and PLTP appear to have roles in generating small HDL important as initial acceptors of cell cholesterol and LCAT substrates during RCT.

LTP occurs also in cerebrospinal (Albers *et al.*, 1992) and follicular (Ravnik *et al.*, 1992) fluid; in the latter case lipid transfer may be involved in sperm capacitation. However, LTP is not essential for this process, as individuals with genetic LTP deficiency are not infertile.

1.4 Mechanism of LTP

1.4.1 Basic Models

Kinetic studies provide two possible models for neutral lipid transfer by LTP. One model proposes that LTP acts as a carrier to shuttle lipids across the aqueous environment from donor to acceptor lipoproteins (Barter and Jones, 1980). A second model proposes that LTP mediates transient formation of a ternary complex between itself and the donor and acceptor lipoproteins, with lipid transfer somehow occurring during existence of this complex (Ihm *et al.*, 1982b). The actual mechanism remains controversial and various findings about LTP, such as its ability to carry neutral lipids provide support for the former hypothesis whereas numerous instances of increased LTP binding to lipoproteins leading to enhanced transfer activity support the latter hypothesis.

1.4.2 Relationship between CE and TG Transfer Activities of LTP

Chemical modification of LTP with the sulfhydryl reagent parachloromercuriphenyl sulfonate (pCMPS) was found to dissociate the neutral lipid transfer activity of LTP, resulting in inhibition of TG transfer in radioisotopic transfer assays to a greater extent than CE transfer (Hopkins and Barter, 1980; Morton and Zilversmit, 1982). Two groups have produced mAbs against LTP which also inhibited TG transfer in radioisotopic assays greater than CE transfer (Fukasawa *et al.*, 1992, Melchior *et al.*, 1995). On the other hand, short chain CE (Busch and Harmony, 1990), PD 140195 (Bisgaier *et al.*, 1994) and 6-chloromercuric cholesterol (Epps *et al.*, 1995a; Melchior *et al.*, 1995), were found to inhibit CE transfer to greater extent in radioisotopic transfer assays. Being able to inhibit the neutral lipid transfer activities of LTP separately lead the workers involved to conclude that CE and TG are transferred by independent activities of LTP.

On the other hand, using reconstituted lipoproteins of defined lipid composition, CE and TG were shown to compete for transfer by LTP, whereas PL transfer occurred independently (Morton and Zilversmit, 1983). According to this study, the relative ratio of CE to TG in the donor lipoprotein determines which lipid will be transferred while the total amount of lipid transferred remains constant. Thus, LTP activity between lipoproteins similar in core mole ratio of CE to TG, i.e. HDL and LDL, results in bidirectional exchange only, whereas between lipoproteins differing in CE to TG ratio, i.e. HDL and VLDL, both bidirectional exchange and net neutral lipid mass transfer by heteroexchange occur (Fig. 1.4, and reviewed in Morton, 1990). Such an inter-relationship between CE and TG transfer activity of LTP could be well accomodated by the scheme of a single neutral lipid binding site within LTP whereby CE and TG compete for binding and transfer (discussed below). Within this model, dissociation of neutral lipid transfer as in inhibition of radioisotopic TG transfer to greater extent than CE transfer seen with pCMPS treatment (Morton and Zilversmit, 1982) or Fab fragments (Swenson *et al.*, 1989) is explained as hindrance of the larger ligand, TG, from entry into the binding site and subsequent transfer (Swenson *et al.*, 1989). This model also implies inhibition of CE and TG mass transfer should occur when LTP is placed under dissociating conditions, and there is some evidence for this (Hopkins and Barter, 1980), although later refuted (Hopkins and Barter, 1982).

Diagrams of the two models of neutral lipid binding and transfer by LTP: independent sites or a single site, are shown in Fig. 1.5.

1.4.3 LTP Distribution in Plasma and Binding to Lipoproteins

Density gradient ultracentrifugation of human plasma shows that LTP is predominantly associated with HDL_3 and smaller HDL (Groener *et al.*, 1984; Marcel *et al.*, 1990). Agarose chromatography provided a similar result, with most of LTP in small HDL in fasting plasma, and a shift to larger HDL and apoB-containing lipoproteins during lipemia (Tall *et al.*, 1986). Immunoaffinity chromatography showed that most of plasma LCAT and LTP are located in the Lp(A-I) fraction of apoA-I-containing particles, although in separate particles of different size (Cheung *et al.*, 1986), while a later report showed that there is significant LTP in Lp(A-I/A-II), especially in hyperlipidemic male plasma (Moulin *et al.*, 1994). One group showed by two-dimensional electrophoresis that a significant portion of plasma LTP migrates as large particles along with LCAT and apoD in a pre-beta fraction of Lp(A-I) (Francone *et al.*, 1989); another group found all LTP to have pre-beta migration but with a particle size too small to accomodate LCAT, apoA-I, D, and LCAT together in one particle (Marcel *et al.*, 1990).

The binding between LTP and HDL isolated by gel filtration can be disrupted by lowering the pH or adding divalent cations (or ultracentrifugation), and seems primarily an electrostatic interaction between LTP and the negative charge of lipoprotein PL phosphate groups (Pattnaik and Zilversmit, 1979). The K_d of LTP binding to immobilized VLDL, LDL, or HDL under steady state conditions was found to be similar (about 25 nM), so while LTP interacts with all lipoproteins, under physiological conditions, with molar concentrations of HDL being much higher than LDL or VLDL, HDL will bind most of the LTP in plasma (Morton, 1985). Rapid gel filtration also demonstrated that LTP binds to all lipoprotein classes, with K_d values of 130 nM, 60 nM, and 30 nM for VLDL, LDL, and HDL, respectively, also predicting that a majority of LTP will be bound to HDL in plasma (Nishida *et al.*, 1993).

While the majority of LTP in human plasma is bound to lipoproteins, in other mammals such as rabbit, plasma LTP is predominantly free (Tall *et al.*, 1987). This may reflect a species difference in LTP or HDL. In transgenic mice expressing human LTP and apoA-I, more LTP was bound to HDL than in transgenic mice expressing LTP alone, which may be due to differences in HDL particles formed by the human apoA-I or in protein sequence of mouse versus human apoA-I (Hayek *et al.*, 1992).

1.4.4 Carrying of Neutral Lipids by LTP

Scatchard analysis of neutral lipid binding to LTP revealed near stoichiometric binding of CE and TG while PC binding was 11 mol/mol of LTP (Swenson *et al.*, 1988). This study demonstrated that LTP could act as a carrier to transfer bound lipid to lipoproteins, and also that pCMPS treatment leads to parallel decreases in TG binding and transfer by LTP. Furthermore, the mechanism of a neutralizing mAb to block all radioisotopic transfer of neutral lipids by LTP was found to be through an inhibition of neutral lipid binding by LTP (Swenson *et al.*, 1989). These results indicate that binding of lipid to LTP is intrinsic to the mechanism of neutral lipid transfer by LTP, whether lipid transfer occurs through a carrier or ternary complex mechanism.

1.5 Regulation of LTP Activity

1.5.1 Neutral Lipid Specificity of LTP

Human LTP shows specificity for CE over TG, while rabbit LTP apparently doesn't (Morton and Zilversmit, 1983). High specificity was reported for pyrene-CE and CE over TG in transfer between lipid microemulsion particles mediated by human LTP (Ohnishi *et al.*, 1994b).

In addition to CE and TG, LTP is able to facilitate transfer of retinyl ester (Morton and Zilversmit, 1982; Zilversmit *et al.*, 1982), cholesteryl ether (Stein *et al.*, 1985, 1986, 1989; Green *et al*, 1989), pyrene-CE (Milner *et al.*, 1991, Dousset and Douste-Blazy, 1992) and -TG (Ohnishi *et al.*, 1994), Cholesteryl 4,4-difluoro-5,7-dimethyl-4-bora-3α,4α-diaza-3-indacene-3-dodecanoate (BODIPY-CE) (Bisgaier *et al.*, 1994, Epps *et al.*, 1995), as well as probucol (M. Tsujita and S. Yokoyama, personal communication) between donor and acceptor lipoproteins. LTP prefers lengthy and saturated acyl chains of CE (Morton, 1986; Green and Pittman, 1991). However, radioisotopic oxidized and unoxidized cholesteryl linoleates were transferred at comparable rates (Christison *et al.*, 1995). Radioisotopic cholesteryl ethers were transferred slower than esters in one report (Green *et al.*, 1989). Short chain cholesteryl ether was also reported to inhibit radioisotopic CE transfer activity of LTP but not TG transfer (Busch and Harmony, 1990). Interestingly, two compounds, PD 140105 (Bisgaier *et al.*, 1994) and 6-chloromercuric cholesterol (Melchior *et al.*, 1995) have structural similarity to cholesterol and specifically inhibit CE transfer.

1.5.2 Modulation of Lipoprotein Composition

Lipolysis *in vitro* was found to stimulate radioisotopic and mass transfer of CE from HDL to VLDL (Tall *et al.*, 1984), due to enhanced LTP binding to lipoproteins produced by the accumulation of fatty acids in lipoproteins (Sammett and Tall, 1985). The *in vivo* significance is unclear, as albumin keeps free fatty acid levels low, but local build-up may occur in tissues active in lipolysis or in certain pathological conditions. Sodium oleate was also reported to disrupt LTP mediated heteroexchange of CE for TG between HDL and VLDL (Barter *et al.*, 1990b) or equilibrium exchange of neutral lipids between HDL and LDL (Barter *et al.*, 1990c), in both cases resulting in formation of small HDL.

Increased free cholesterol content of lipoproteins in diabetic plasma was reported to inhibit LTP activity (Fielding *et al.*, 1984). More recently, increasing the free cholesterol content of lipoproteins was found to increase the rate of mass transfer of CE from HDL to VLDL, by reducing the back-transfer of CE from VLDL (Morton, 1988) without influencing extent of LTP binding to lipid particles (Rajaram *et al.*, 1994). The mechanism of the free cholesterol effect may be through an influence on neutral lipid concentration in the lipoprotein PL surface (Morton and Steinbrunner, 1990). Stachybotramide, a multi-ringed fungal compound resembling cholesterol, probably has similar effects on CE mass transfer by reducing back-transfer of CE from VLDL to HDL (Takahashi *et al.*, 1995).

Prostaglandin E_1 increased both spontaneous and LTP mediated radioisotopic CE transfer from HDL to LDL, with re-organization of lipoprotein surface the suggested mechanism (Muzya *et al.*, 1987). Oxidized LDL was shown to have higher affinity for LTP than native LDL (Zawadski *et al.*, 1991), possibly through generation of lipoperoxides leading to increased negative charge of lipoprotein surface (Hesler *et al.*, 1987).

1.5.3 Effects of Proteins

Protein inhibitors of radioisotopic neutral lipid transfer by LTP have been described in humans and species (rat, pig) lacking plasma LTP activity (Morton and Zilversmit, 1981b; Tollefson *et al.*, 1988b) with human forms having an M_r of approximately 32,000 and acidic pI (Morton and Zilversmit, 1981b; Son and Zilversmit, 1984; Nishide *et al.*, 1989). The inhibitor was reportedly associated with a subclass of HDL (Nishide *et al.*, 1989) while another study found it primarily in LDL (Morton and Green, 1994). Inhibition could be overcome by increasing lipoprotein concentration (Son and Zilversmit, 1984), suggesting that the inhibition occurs at the lipoprotein surface. Recently, it was reported that the inhibitor preferentially diminishes transfers involving LDL (Morton and Green, 1994).

When certain baboons are fed a high-fat, high cholesterol diet, high levels of HDL accumulate, associated with lower radioisotopic CE transfer activity (Kushwaha *et al.*, 1990). A plasma inhibitor of LTP activity which is elevated in these animals corresponded to the N-terminal 38 residues of apoC-I (Kushwaha *et al.*, 1993). This study also showed that this inhibitor forms non-covalent associations with apoA-I and E and suggested that it arises from post-translational proteolysis in liver or plasma.

Exchangeable apolipoproteins stimulate transfer of a fluorescent CE analog between lipid microemulsion particles in a dose-dependent manner proportional to saturation of the particle surface (Milner *et al.*, 1991; Ohnishi *et al.*, 1993). Enhanced movement of neutral lipids to the particle surface was the suggested mechanism. ApoA-I was found to be inhibitory to radioisotopic neutral lipid transfer from LDL to HDL by LTP *in vitro* (Son and Zilversmit, 1984). Using similar methods, others found that apoA-I and E had little effect while apoC-III and A-II stimulated LTP activity (Sparks *et al.*, 1991). Another group found that apo A-I, A-II, and A-IV all modulated LTP activity, with apo A-II being distinctly inhibitory; LTP complexes with apoA-I or A-II were also demonstrated (Guyard-Dangremont, 1994). Introduction of apoE to VLDL from an apoE deficiency patient lead to increased LTP activity when used as an acceptor in a radioisotopic assay, and also increased LTP binding to VLDL (Kinoshita *et al.*, 1993). ApoA-II was found to inhibit LTP mediated HDL conversion (Rye *et al.*, 1992), while apoA-IV increased this process (Lagrost *et al.*, 1990). However, transgenic expression of apoA-II in mice did not inhibit neutral lipid transfer *in vivo* (Zhong *et al.*, 1994).

The affinity of LTP for HDL is reportedly increased by the presence of LCAT (Nishida *et al.*, 1990), consistent with the report of LTP existing in plasma as a complex along with apoA-I, D, and LCAT (Francone *et al.*, 1989). Radioisotopic CE transfer between Lp(a) and HDL was found to be slower than between LDL and HDL (Groener and Kostner, 1987), due to either hindrance of LTP binding or effect of apo(a) on lipoprotein surface properties (reviewed in Lagrost, 1994).

1.5.4 Relative Levels of LTP and Lipoproteins

The approximate plasma concentrations of VLDL, LDL, and HDL are 0.067, 1.87, and 12.8 μM, respectively, while of LTP is about 60 nM (Morton, 1985). Increased amounts of TG-rich acceptor lipoproteins contributed to increased rates of radioisotopic and mass CE transfer from HDL to these acceptors in incubated plasma during lipemia (Tall *et al.*, 1986). During incubation of normolipidemic plasma, there is a strong correlation between net CE transfer to VLDL and VLDL TG levels (Yen *et al.*, 1989; Mann *et al.*, 1991). Supplementation of normal plasma with VLDL was able to stimulate net CE transfer to VLDL while addition of LTP did not; with hypertriglyceridemic plasma, LTP addition did increase net CE transfer (Mann *et al.*, 1991). Thus, VLDL concentration determines net CE transfer rate in normal human subjects, while LTP is rate limiting as VLDL concentrations increase, as in hypertriglyceridemia.

1.6 Structure-Function Relationships of LTP

1.6.1 Stability and Protein Folding of LTP

LTP is resistant to inactivation by protease digestion or denaturants (Hesler *et al.*, 1989), which along with its resistance to thermal inactivation indicate a highly stable tertiary protein structure. This study also showed that although a small, active domain of LTP was reportedly isolated (Busch *et al.*, 1987), none could be produced by expression of LTP cDNA in E. coli. The human LTP cDNA sequence codes for an extremely hydrophobic protein with a predicted alpha-helical content of 35% using the algorithm of Garnier, Osguthorpe, and Robson (Drayna *et al.*, 1987a). Members of the LPS binding protein family have a predominance of basic residues in their N-terminal half while the more hydrophobic C-terminal portion contains more acidic residues. LPS binding and membrane anchoring functions of bactericidal permeability increasing protein have been ascribed to the N- and C-terminal portions, respectively, and by analogy, the lipoprotein binding and neutral lipid binding functions of LTP may be similarly divided (reviewed in Tall, 1993). Linker insertion scanning mutagenesis of LTP revealed certain critical regions where insertional alterations impaired neutral lipid transfer activity, presumably due to local perturbations of secondary structure (Wang *et al.*, 1991).

1.6.2 Neutral Lipid Binding Site of LTP

The epitope of TP2, a mAb which blocks all radioisotopic (Hesler *et al.*, 1988) and mass (Yen *et al.*, 1989) transfer of neutral lipid by LTP was localized to the C-terminal 13 amino acids of LTP (Swenson *et al.*, 1989, Wang *et al.*, 1992). Deletion of this region results in impaired neutral lipid transfer activity by LTP while PL transfer was retained (Wang *et al.*, 1992). The last 11 amino acids of LTP may form an amphipathic helix and point mutagenesis revealed that hydrophilic amino acids were necessary for TP2 binding whereas hydrophobic ones were required for CE transfer (Wang *et al.*, 1993). Deletion of the last 6 amino acids of LTP produced an LTP inactive in CE transfer and CE binding while lipoprotein binding was unimpaired (Wang *et al.*, 1995), further support for the role of the LTP C-terminal in formation of a hydrophobic pocket involved in neutral lipid binding and transfer (reviewed in Tall, 1993). It is also unknown which Cys is modified by pCMPS preventing neutral lipid binding (Swenson *et al.*, 1988) and there are no Cys residues in the TP2 epitope region described above (Drayna *et al.*, 1987a).

1.6.3. Lipoprotein Binding Site of LTP

Binding of TP2 Fab fragments to LTP leads to increased binding of LTP to lipoproteins, supposedly through mimicking of ligand binding to LTP, inducing a conformation change in the site involved in lipoprotein binding (Swenson *et al.*, 1989). A Lys triplet beginning at residue 378 of human LTP may be involved in electrostatic interaction with lipoprotein PL headgroups and insertional alterations in the region of residues 373-379 lead to impaired neutral lipid transfer activity (Wang *et al.*, 1991). Overlapping deletions of human LTP identify the region between residues 451-458 to be necessary for lipoprotein binding and lipid transfer activity, as well as conforming to the consensus sequence for an octapeptide found in many enzymes and proteins whose functions involve binding of non-polar lipids (Au-young and Fielding, 1992).

1.6.4. Glycosylation of LTP

Neuraminidase or glycosidase F treatment of LTP did not abolish CE transfer activity while tunicamycin treatment of cells prevented secretion (Swenson *et al.*, 1987). However, another group reported inactivation of neuraminidase was necessary to maintain activity of an LTP preparation (Kato *et al.*, 1989). Recently, deglycosylation of rabbit LTP expressed in yeast was shown to abolish lipid transfer activity (Kotake *et al.*, 1996). The 4 potential sites for Asn-linked glycosylation of LTP all undergo post-translational modification with variable glycosylation (about 50% of LTP in human plasma) at one site, residue 341 (Stevenson *et al.*, 1993). This study also showed that glycosylation of two sites, residues 88 and 396, were important for LTP secretion, and while loss of the site at residue 341 resulted in LTP with higher lipid transfer specific activity, wild type levels of activity were retained with individual loss of the other sites.

1.6.5 Alternative Splicing and Mutations of LTP

Co-expression of full length and exon 9-deleted LTP in COS cells lead to poor secretion of the shorter form and inhibited secretion of the full-length form (Quinet *et al.*, 1993b), and it was suggested that alternative splicing of LTP mRNA (Inazu *et al.*, 1992) regulates LTP secretion by directing synthesis of a poorly secreted protein which forms heteromers with LTP leading to retention of both in the endoplasmic reticulum.

Expression of a mutant LTP with residue 442 changed from Asp to Gly, corresponding to a prevalent missense allele in humans (Inazu *et al.*, 1994), was shown to result in a moderately secreted protein with reduced lipid transfer specific activity compared to wild type (Takahashi *et al.*, 1993). It was also shown that co-expression of mutant and wild type LTP resulted in secretion of LTP with lower specific activity than expected for equimolar amounts of wild type and mutant proteins; it was suggested that the mutant LTP dominantly inhibited wild type LTP by formation of heteromeric complexes within the secretory pathway. These results also implied that the active form of LTP is multimeric and evidence for this was provided by quasielastic light scattering.

1.7 Scope of Thesis

In order to characterize further the mechanism and regulation of LTP activity, the following studies were carried out: (1) A fluorescence assay for monitoring LTP mediated transfer of pyrene labeled CE between lipid microemulsion particles was established and used to characterize regulation of LTP activity by exchangeable apolipoproteins; (2) LTP was isolated from rabbit plasma and its interaction with lipid microemulsion particles was characterized; (3) novel mAbs were generated against rabbit LTP and their effects on LTP activity and behavior highlighted important features of the mechanism of neutral lipid transfer by LTP.

Table 1.1. Composition of major lipoproteins[a]

Lipoprotein Class	Percent Dry Weight as		Number of Molecules per Particle	
	Lipid	Protein	TG	CE
Chylomicron	98	2	507,000	27,700
VLDL	92	8	11,500	3,600
LDL	79	21	298	1,310
HDL	45-59	41-55	10-19	32-90

[a] From Shen *et al.*, 1977

Table 1.2. Chronological list of reports on purification and description of plasma lipid transfer factor(s)

Reference	Transfer Activity	M_r (method)	Further Details Within Report
Chajek and Fielding, 1978	CE, TG	35 K (SDS-PAGE)	HDL associated, antibodies against factor remove transfer activity from human plasma, possibly related to apoD
Pattnaik *et al.*, 1978	CE	80 K (SDS-PAGE)	human plasma glycoprotein, pI = 5, distinct from known apolipoproteins, no LCAT activity
Ihm *et al.*, 1980	CE, PC	150 K (gel filtration)	human has CE, PC transfer activity, rat only PC transfer
Rajaram *et al.*, 1980	TG	115 K (gel filtration)	rabbit serum glycoprotein, pI = 9
Ihm *et al.*, 1982a	CE, PC	58, 63 K (SDS-PAGE); 61 K (urea gel)	human plasma lipid transfer complex (LTC), in complex with LCAT, heat resistant, rat and cow only transfer PC
Morton and Zilversmit, 1982	CE, TG, retinyl ester, cholesteryl ether, PC	58, 66 K (gel filtration)	CE and TG transfer activities co-purify, TG transfer activity inhibited by pCMPS, CE transfer unaffected
Abbey *et al.*, 1984	CE, TG, PC	68 K (SDS-PAGE)	rabbit plasma protein, change pig lipoprotein composition
Albers *et al.*, 1984	CE, TG, PC	64 K (SDS-PAGE); 65 K (gel filtration)	human plasma protein, pI = 5, heat resistant, non-heparin binding, another protein, heat sensitive, transfers PC
Ierides *et al.*, 1985	CE	70 K (SDS-PAGE)	rabbit plasma protein, pI = 5.95, functional unit is monomer by radiation inactivation
Ogawa and Fielding, 1985	CE	60 K (gel filtration)	human plasma protein in complex with LCAT and apoD
Bastiras and Calvert, 1986	CE	66 K (SDS-PAGE)	human lipid transfer protein (LTP-I) isolated by FPLC

(continued)

Busch *et al.*, 1986	CE, TG	67, 58, 150 K (SDS-PAGE)	human lipid transfer complex (LTC) isolated by immunoaffinity chromatography, pI = 5.2 and 8.7
Busch *et al.*, 1987	CE, TG, PC	65 K (SDS-PAGE); 18, >150 K (gel permeation)	LTC proteins resolved by HPLC, resulting in transfer protein (TP) with a catalytic domain (Cd) and heparin-binding domain (Hd).
Hesler *et al.*, 1987	CE, TG, PC	74 K (SDS-PAGE); 140, 440 K (Native-PAGE)	human plasma cholesteryl ester transfer protein (CETP) with pI = 5.3, 5.6, high content of non polar amino acids
Jarnagin *et al.*, 1987	CE, TG	74 K (SDS-PAGE)	human CETP, pI = 5.2, rich in hydrophobic amino acids, N-terminal sequence similar to Hesler *et al.*, (1987)
Tollefson *et al.*, 1988a	PC	69 K (SDS-PAGE); 70 K (gel filtration)	human plasma PL transfer protein II (LTP-II), immunologically distinct from LTP-I, pI = 5.0
Kato *et al.*, 1989	CE	64 K (SDS-PAGE)	human LTP is many pI isoforms 4.6 - 5.2 by variable sialation, N-terminal sequence as by Hesler *et al.*, (1987)
Ohnishi *et al.*, 1990	CE, TG	66, 69 K (SDS-PAGE)	both bands have same N-terminal sequence, anomolous behavior during gel filtration chromatography
Ravnik *et al.*, 1992	CE	64 K (gel filtration)	human follicular fluid contains LTP-I, which can stimulate sperm capacitation
Jauhiainen *et al.*, 1993	PC	75 K (SDS-PAGE)	human PL transfer protein (PLTP) causes HDL conversion, not inhibited by mAb against CETP
Ko *et al.*, 1993	CE	74 K (SDS-PAGE); 83 K (gel permeation)	rabbit LTP with multiple pI in range 5.7-5.9, CD spectroscopy indicates high beta structure content, protein binds strongly to lipid microemulsion particles

	Chylomicron	VLDL	LDL	HDL
Density (g/mL)	< 0.98	< 1.006	1.006-1.063	1.063-1.21
Diameter (nm)	> 120	> 40	20	8-12

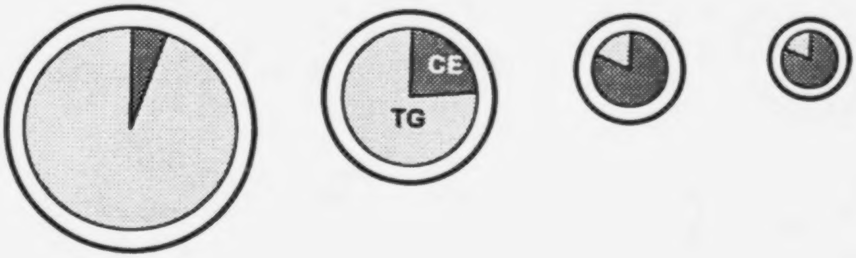


Figure 1.1. Lipoprotein structure and core lipid ratio. The densities and sizes of the major lipoprotein classes are listed. Lipoproteins are not drawn to scale, and apolipoproteins have been omitted for simplicity. Depicted are spherical lipoproteins consisting of a surface monolayer of PL and free cholesterol (white), surrounding a core of neutral lipids TG (light grey) and CE (dark grey). The proportion of CE to TG within the cores were derived from data within Shen *et al.*, 1977.

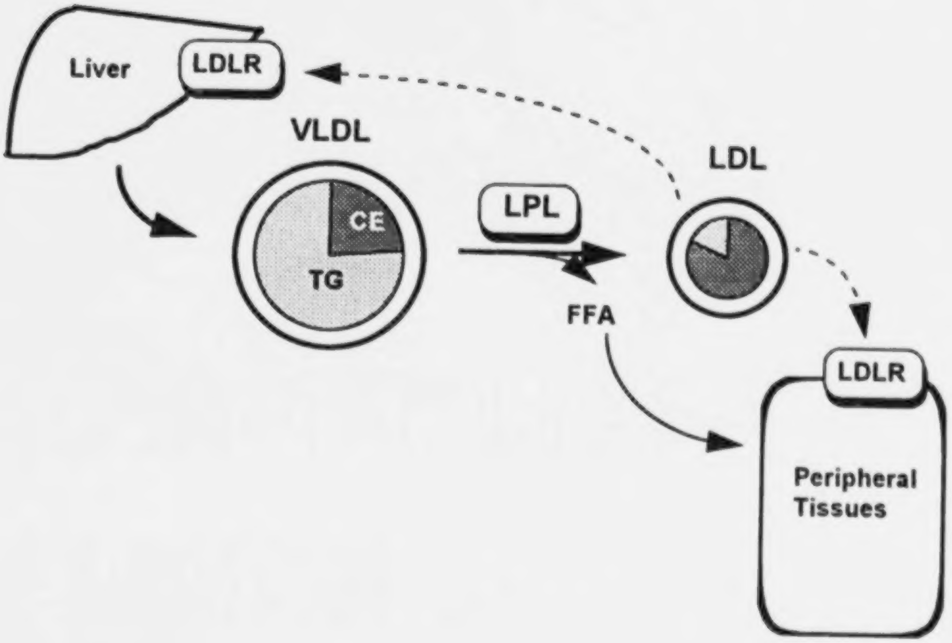


Figure 1.2. Metabolism of hepatic TG-rich lipoproteins. Following secretion by the liver, VLDL are acted on by lipoprotein lipase (LPL) to liberate free fatty acids (FFA) from the glycerolipids. These free fatty acids are taken up for energy production or storage by the peripheral tissues, and continued lipolysis results in formation of LDL. LDL are cleared from circulation mainly by liver LDLR, while certain peripheral tissues with high sterol demands will also employ LDLR to acquire LDL as a sterol source (forward cholesterol transport).

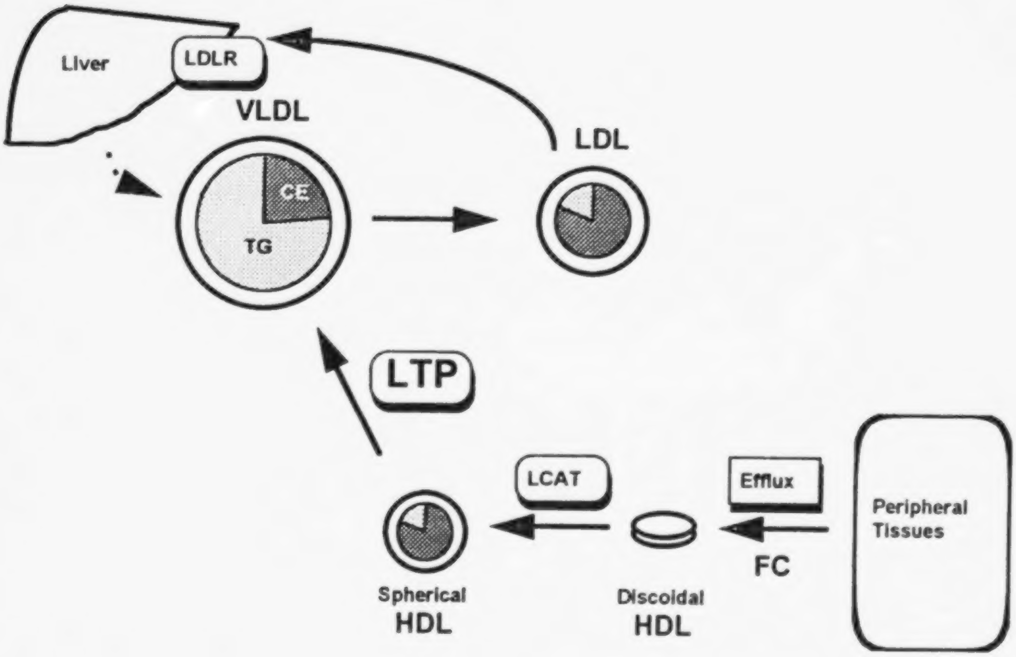

Liver
LDLR
VLDL
CE
TG
LDL
LTP
LCAT
Efflux
Peripheral Tissues
FC
Spherical HDL
Discoidal HDL

Figure 1.3. HDL metabolism and the postulated RCT pathway involving LTP. Spherical HDL are formed by efflux of free cholesterol (FC) to discoidal HDL and LCAT action. LTP mediates net transfer of HDL CE to TG-rich lipoproteins such as VLDL. Clearance of VLDL remnants and LDL by the liver completes a hypothetical pathway for the return of excess peripheral tissue cholesterol back to the liver, the only organ capable of catabolism and elimination of cholesterol from the body.

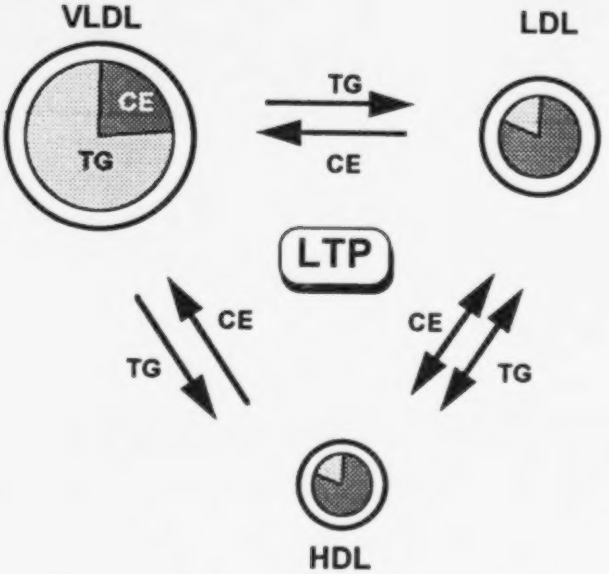
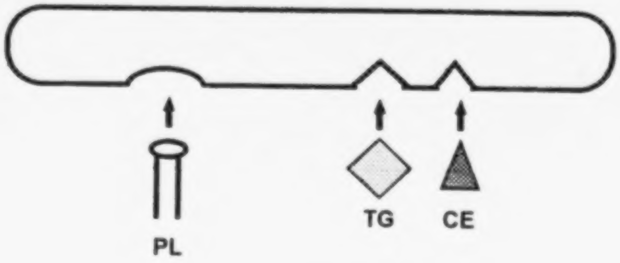


Figure 1.4. Effects of LTP on lipoprotein lipid composition. LTP facilitates bidirectional homoexchange of neutral lipids between all classes (not shown). Heteroexchange of neutral lipids between lipoproteins similar in core ratio of CE to TG, such as LDL and HDL, also occurs with the net effect of both processes being exchange, (denoted by double headed arrows). Heteroexchange between lipoproteins differing in core ratios of CE to TG, for instance between LDL or HDL and VLDL, results in net transfer of CE from LDL and HDL, which are CE-rich, to VLDL and net transfer of TG from TG-rich VLDL in the opposite direction (denoted by single headed arrows).

Independent sites



Single site

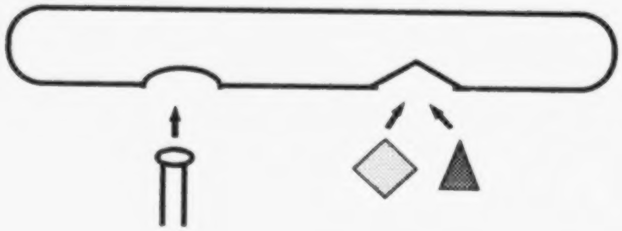


Figure 1.5. Models of neutral lipid binding and transfer by LTP. Schematic diagrams of the two different models are shown. In the independent site model, TG, CE, and PL all bind to distinct sites within LTP before transfer. In the single site model, TG and CE binding is mutually exclusive to a shared site while PL binds to a separate site within LTP.

1.8 References

Abbey, M., and Calvert, G.D. (1989) Effects of blocking plasma lipid transfer protein activity in the rabbit. *Biochim. Biophys. Acta* 1003, 20-29

Abbey, M., Calvert, G.D., and Barter, P.J. (1984) Changes in lipid and apolipoprotein composition of pig lipoproteins facilitated by rabbit lipid transfer protein. *Biochim. Biophys. Acta* 793, 471-480

Abbey, M., Clifton, P., Kestin, M., Belling, B., and Nestel, P. (1990) Effect of fish oil on lipoproteins, lecithin:cholesterol acyltransferase, and lipid transfer protein activity in humans. *Atherosclerosis* 10, 85-94

Acton, S., Rigotti, A., Landschulz, K.T., Xu, S., Hobbs, H. *et al.* (1996) Identification of scavenger receptor SR-BI as a high density lipoprotein receptor. *Science* 271, 518-520

Agellon, L.B., Quinet, E.M., Gillette, T.G., Drayna, D.T., Brown, M.L. *et al.* (1990) Organization of the human cholesteryl ester transfer protein gene. *Biochemistry* 29, 1372-1376.

Agellon, L.B., Walsh, A., Hayek, T., Moulin, P., Jiang, X.C. (1991) Reduced high density lipoprotein cholesterol in human cholesteryl ester transfer protein transgenic mice. *J. Biol. Chem.* 266, 10796-10801

Agellon, L.B., Zhang, P., Jiang, X.C., Mendelsohn, L., and Tall, A.R. (1992) The CCAAT/enhancer-binding protein trans-activates the human cholesteryl ester transfer protein gene promoter. *J. Biol. Chem.* 267, 22336-22339

Albers, J.J., Tollefson, J.H., Chen, C.-H., and Steinmetz, A. (1984) Isolation and characterization of human plasma lipid transfer proteins. *Arterioscler.* 4, 49-58

Albers, J.J., Tollefson, J.H., Wolfbauer, G., and Albright Jr., R.E. (1992) Cholesteryl ester transfer protein in human brain. *Int. J. Clin. Lab. Res.* 21, 264-266

Albers, J.J., Wolfbauer, G., Cheung, M.C., Day, J.R., Ching, A.F.T. *et al.* (1995) Functional expression of human and mouse plasma phospholipid transfer protein: effect of recombinant and plasma PLTP on HDL subspecies. *Biochim. Biophys. Acta* 1258, 27-34

Akanuma, Y., and Glomset, J.A. (1968) *In vitro* incorporation of cholesterol-^{14}C into very low density lipoprotein cholesteryl esters. *J. Lipid Res.* 620-626

Ahnadi. C.-E., Berthezene, F., and Ponsin, G. (1993) Simvistatin-induced decrease in the transfer of cholesterol esters from high density lipoproteins to very low density lipoproteins in normolipidemic subjects. *Atherosclerosis* 99, 219-228

Arai, T., Yamashita, S., Hirano, K., Sakai, N., Kotani, K. *et al.* (1994) Increased plasma cholesteryl ester transfer protein in obese subjects. A possible mechanism for the reduction of serum HDL cholesterol levels in obesity. *Arterioscl. Thromb.* 14, 1129-1136

Au-Young, J., and Fielding, C.J. (1992) Synthesis and secretion of wild-type and mutant human plasma cholesteryl ester transfer protein in baculovirus-transfected insect cells: the carboxyl-terminal region is required for both lipoprotein binding and catalysis of transfer. *Proc. Natl. Acad. Sci. USA* 89, 4094-4098

Bagdade, J.D., Lane, J.T., Stone, N., Ritter, M.C., and Subbaiah, P.V. (1990) Persistent abnormalities in lipoprotein composition and cholesteryl ester transfer following lovastatin treatment. *J. Lipid Res.* 31, 1263-1269

Bagdade, J.D., Ritter, M.C., and Subbaiah, P.V. (1991a) Accelerated cholesteryl ester transfer in plasma of patients with hypercholesterolemia. *J. Clin. Invest.* 87, 1259-1265.

Bagdade, J.D., Ritter, M.C., and Subbaiah, P.V. (1991b) Accelerated cholesteryl ester transfer in plasma of patients with insulin-dependent diabetes mellitus. *Eur. J. Clin. Invest.* 21, 161-167

Bagdade, J.D., Ritter, M.C., Davidson, M., and Subbaiah, P.V. (1992) Effect of marine lipids on cholesteryl ester transfer and lipoprotein composition in patients with hypercholesterolemia. *Arterioscl. Thromb.* 12, 1146-1152

Bagdade, J.D., Lane, J.T., Subbaiah, P.V., Otto, M.E., and Ritter, M.C. (1993) Accelerated cholesteryl ester transfer in noninsulin-dependent diabetes mellitus. *Atherosclerosis* 104, 69-77

Barter, P.J., and Jones, M.E. (1979) Rate of exchange of esterified cholesterol between human plasma low and high density lipoproteins. *Atherosclerosis* 34, 67-74

Barter, P.J., and Jones, M.E. (1980) Kinetic studies of the transfer of esterified cholesterol between human plasma low and high density lipoproteins. *J. Lipid Res.* 21, 238-249

Barter, P.J., and Lally, J.I. (1978) The activity of an esterified cholesterol transferring factor in human and rat serum. *Biochim. Biophys. Acta* 531, 233-236

Barter, P.J., and Lally, J.I. (1979) *In vitro* exchanges of esterified cholesterol between serum lipoprotein fractions: studies of humans and rabbits. *Metabolism* 28, 230-236

Barter, P.J., Lally, J.I., and Wattchow, D. (1979) Metabolism of triglyceride in rabbit plasma low and high density lipoproteins: studies *in vivo* and *in vitro*. *Metabolism* 28, 614-618

Barter, P.J., Rajaram, O.V., Chang, L.B.F., Rye, K.A., Gambert, P. *et al.* (1988) Isolation of a high-density-lipoprotein conversion factor from human plasm. A possible role of apolipoprotein A-IV as its activator. *Biochem. J.* 254, 179-184

Barter, P.J., Chang, L.B.F., Newnham, H.H., K.-A. Rye, and Rajaram, O.V. (1990a) The interaction of cholesteryl ester transfer protein and unesterified fatty acids promotes a reduction in particle size of high-density lipoproteins. *Biochim. Biophys. Acta* 1045, 81-89

Barter, P.J., Chang, L.B.F., and Rajaram, O.V. (1990b) Sodium oleate dissociates the heteroexchange of cholesteryl esters and triacylglycerol between HDL and triacylglycerol-rich lipoproteins. *Biochim. Biophys. Acta* 1047, 294-297

Barter, P.J., Chang, L.B.F., and Rajaram, O.V. (1990c) Sodium oleate promotes a redistribution of cholesteryl esters from high to low density lipoproteins. *Atherosclerosis* 84, 13-24

Bastiras, S., and Calvert, G.D. (1986) Purification of human plasma lipid transfer protein using fast protein liquid chromatography. *J. Chromatogr.* 383, 27-34

Berg, K., Kondo, I., Drayna, D., and Lawn, R. (1989) "Variability gene" effect of cholesteryl ester transfer protein (CETP) genes. *Clin. Genet.* 35, 437-445

Bisgaier, C.L., Siebenkas, M.V., Brown, M.L., Inazu, A., Koizumi, J. (1991) Familial cholesteryl ester transfer protein deficiency is associated with triglyceride-rich low density lipoproteins containing cholesteryl esters of probable intracelluar origin. *J. Lipid Res.* 32, 21-33

Bisgaier, C.L., Essenburg, A.D., Minton, L.L., Homan, R., Blankley, C.B. *et al.*, (1994) Cholesteryl ester transfer protein inhibition by PD 140195. *Lipids* 29, 811-818

Blake, W.L., Ulrich, R.G., Marotti, K.R., and Melchior, G.W. (1994) The development of fatty liver is accelerated in transgenic mice expressing cynomolgus monkey cholesteryl ester transfer protein. *Biochem. Biophys. Res. Commun.* 205, 1257-1263

Brown, M.S., and Goldstein, J.L. (1986) A receptor-mediated pathway for cholesterol homeostasis. *Science* 232, 34-47

Brown, M.S., Kovanen, P.T., and Goldstein, J.L. (1981) Regulation of plasma cholesterol by lipoprotein receptors. *Science* 212, 628-635

Brown, M.L., Inazu, A., Hesler, C.B., Agellon, L.B., Mann, C. *et al.* (1989) Molecular basis of lipid transfer protein deficiency in a family with increased high-density lipoproteins. *Nature* 342, 448-451

Brown, G., Albers, J.J., Fisher, L.D., Schaefer, S.M., Lin, J.-T., *et al.* (1990) Regression of coronary artery disease as a result of intensive lipid-lowering therapy in men with high levels of apolipoprotein B. *N. Engl. J. Med.* 323, 1289-1298

Busch, S.J., and Harmony, J.A.K. (1990) Cholesteryl ester analogs inhibit cholesteryl ester but not triglyceride transfer catalyzed by the plasma cholesteryl ester-triglyceride transfer protein. *Lipids* 25, 216-220

Busch, S.J., Duvic, C.R., Ellsworth, J.L., Ihm, J., and Harmony, J.A.K. (1986) Immunoaffinity purification of the lipid transfer protein complex directly from human plasma. *Anal. Biochem.* 153, 178-188

Busch, S.J., Stuart, W.D., Hug, B., Mao, S.J.T., and Harmony, J.A.K. (1987) Plasma cholesteryl ester-triglyceride transfer protein. The catalytic domain is a low molecular weight proteolipid. *J. Biol. Chem.* 262, 17563-17571

Chajek, T., and Fielding, C.J. (1978) Isolation and characterization of a human serum cholesteryl ester transfer protein. *Proc. Natl. Acad. Sci. USA* 75, 3445-3449

Cheung, M.C., Wolf, A.C., Lum, K.D., Tollefson, J.H., and Albers, J.J. (1986) Distribution and localization of lecithin:cholesterol acyltransferase and cholesteryl ester transfer activity in A-I-containing lipoproteins. *J. Lipid Res.* 27, 1135-1144

Cheung, M.C., Austin, M.A., Moulin, P., Wolf, A.C., Cryer, D. *et al.* (1993) Effects of pravastatin on apolipoprotein-specific high density lipoprotein subpopulations and low density lipoprotein subclass phenotypes in patients with primary hypercholesterolemia. *Atherosclerosis* 102, 107-119

Chiesa, G., Michelagnoli, S., Cassinotti, M., Gianfranceschini, G., Werba, J. *et al.* (1993) Mechanisms of high-density lipoprotein reduction after probucol treatment: changes in plasma cholesterol esterification/transfer and lipase activities. *Metabolism* 42, 229-235

Christison, J.K., Rye, K.-A., and Stocker, R. (1995) Exchange of oxidized cholesteryl linoleate between LDL and HDL mediated by cholesteryl ester transfer protein. *J. Lipid Res.* 36, 2017-2026

Clay, M.A., Newnham, H.H., Forte, T.M., and Barter, P.I. (1992) Cholesteryl ester transfer protein and hepatic lipase activity promote shedding of apo A-I from HDL and subsequent formation of discoidal HDL. *Biochim. Biophys. Acta* 1124, 52-58

Davis, R.A. (1991) Lipoprotein structure and secretion. In *Biochemistry of lipids, lipoproteins and membranes* (Vance, D.E., and Vance, J., eds) Elsevier Science Publishers, New York, NY, 403-426

Day, J.R., Albers, J.J., Lofton-Day, C.E., Gilbert, T.L., Ching, A.F.T. *et al.* (1994) Complete cDNA encoding human phospholipid transfer protein from human endothelial cells. *J. Biol. Chem.* 269, 9388-9391

Deckelbaum, R.J., Eisenberg, S., Oschry, Y., Granot, E., Sharon, H., and Bengtsson-Olivecrona, G. (1986) Conversion of human plasma high density lipoprotein-2 to high density lipoprotein-3. Roles of neutral lipid exchange and triglyceride lipase. *J. Biol. Chem.* 261, 5201-5208

Dietschy, J.M., Turley, S.D., and Spady, D.K. (1993) Role of liver in the maintenance of cholesterol and low density lipoprotein homeostasis in different animal species, incuding humans. *J. Lipid Res.* 34, 1637-1659

Dolphin, P.J. (1992) Lipolytic enzymes and the role of apolipoproteins in the regulation of their activity. In *Structure and function of apolipoproteins* (Rosseneu, M., ed.) CRC Press, Boca Raton, FL, 295-362

Dousset, N., and Douste-Blazy, L. (1992) Fluorescent determination of cholesteryl ester transfer protein (CETP) activity in plasma. *Clin. Chem.* 38, 306

Drayna, D., Jarnagin, A.S., McLean, J., Henzel, W., Kohr, W. *et al.* (1987a) Cloning and sequencing of human cholesteryl ester transfer protein cDNA. *Nature* 327, 632-634

Drayna, D., and Lawn, R. (1987b) Multiple RFLPs at the human cholesteryl ester transfer protein (CETP) locus. *Nucleic Acids Res.* 15, 4698

Dullaart, R.P.F., Groener, J.E.M., Dikkeschei, L.D., Erkelens, D.W., and Dorenbos, H. (1989) Increased cholesteryl ester transfer activity in complicated Type 1 (insulin-dependent) diabetes mellitus - its relationship with serum lipids. *Diabetologia* 32, 14-19

Dullaart, R.P.F., Hoogenberg, K., Groener, J.E.M., Dikkeschei, L.D., Erkelens, D.W. *et al.* (1990) The activity of cholesteryl ester transfer protein is decreased in hypothyroidism: a possible contribution to alterations in high-density lipoproteins. Eur. *J. Clin. Invest.* 20, 581-587

Dullaart, R.P.F., Sluiter, W.J., Dikkeschei, L.D., Hoogenberg, K., and van Tol, A. (1994a) Effects of adiposity on plasma lipid transfer protein activities: a possible link between insulin resistance and high density lipoprotein metabolism. *Eur. J. Clin. Invest.* 24, 188-194

Dullaart, R.P.F., Hoogenberg, K., Dikkeschei, B.D., and van Tol, A. (1994b) Higher plasma lipid transfer protein activities and unfavorable lipoprotein changes in cigarette-smoking men. *Arterioscl. Thromb.* 14, 1581-1585

Duverger, N., Rader, D., Ikewaki, K., Nishiwaki, M., Sakamoto, T. *et al.* (1995) Characterization of high-density apolipoprotein particles A-I and A-I: A-II isolated from humans with cholesteryl ester transfer protein deficiency. *Eur. J. Biochem.* 227, 123-129

Ellsworth, J.L., Kashyap, M.L., Jackson, R.., and Harmony, J.A.K. (1987) Human plasma lipid transfer protein catalyzes the speciation of high density lipoproteins. *Biochim. Biophys. Acta* 918, 260-266

Epps, D.E., Greenlee, K.A., Harris, J.S., Thomas, E.W., Castle, C.K. *et al.* (1995a) Kinetics and inhibition of lipid exchange catalyzed by plasma cholesteryl ester transfer protein (lipid transfer protein). *Biochemistry* 34, 12560-12569

Epps, D.E., Harris, J.S., Greenlee, K.A., Fisher, J.F., Marschke, C.K. *et al.* (1995b) Method for measuring the activities of cholesteryl ester transfer protein (lipid transfer protein). *Chem. Phys. Lipids* 77, 51-63

Faust, R.A., and Albers, J.J. (1988) Regulated vectorial secretion of cholesteryl ester transfer protein (LTP-I) by the CaCo-2 model of human enterocyte epithelium. *J. Biol. Chem.* 263, 8786-8789

Faust, R.A., Cheung, M.C., and Albers, J.J. (1989) Secretion of cholesteryl ester transfer protein-lipoprotein complexes by human HepG2 hepatocytes. *Atherosclerosis* 77, 77-82

Faust, R.A., Tollefson, J.H., Chait, A., and Albers, J.J. (1990) Regulation of LTP-I secretion from monocyte-derived macrophages by differentiation and cholesterol accumulation *in vitro*. *Biochim. Biophys. Acta* 1042, 404-409

Fazio, S., Marotti, K.R., Lee, Y.-L., Castle, C.K., Melchior, G.W. *et al.* (1994) Co-expression of cholesteryl ester transfer protein and defective apolipoprotein E in transgenic mice alters plasma cholesterol distribution. Implications for the pathogenesis of type III hyperlipoproteinemia. *J. Biol. Chem.* 269, 32368-32372

Fielding, C.J., and Fielding, P.E. (1995) Molecular physiology of reverse cholesterol transport. *J. Lipid Res.*. 36, 211-228

Fielding, C.J., Reaven, G.M., Liu, G., and Fielding, P. (1984) Increased free cholesterol in plasma low and very low density lipoproteins in non-insulin-dependent diabetes mellitus: its role in the inhibition of cholesteryl ester transfer. *Proc. Natl. Acad. Sci. USA* 81, 2512-2516

Foger, B., Wolhfarter, T., Ritsch, A., Lechleitner, M., Milller, C.H. *et al.* (1994) Kinetics of lipids, apolipoproteins, and cholesteryl ester transfer protein in plasma after a bicycle marathon. *Metabolism* 43, 633-639

Franceschini, G., Sirtori, M., Vaccarino, V., Gianfranceschini, G., Rezzonico, L. *et al.* (1989) Mechanisms of HDL reduction after probucol. Changes inHDL subfractions and increased reverse cholesteryl ester transfer. *Arteriosclerosis* 9, 462-469

Francis, G.A. (1995) Secondary dyslipidemias. *Issues Lipidol.* 4, 1-10

Francone, O.L., Gurakar, A., and Fielding, C. (1989) Distribution and functions of lecithin:cholesterol acyltransferase and cholesteryl ester transfer protein in plasma lipoproteins. Evidence for a functional unit containing these activities together with apolipoproteins A-I and D that catalyzes the esterification and transfer of cell-derived cholesterol. *J. Biol. Chem.* 264, 7066-7072

Freeman, D.J., Packard, C.J., Shepherd, J., Gaffney, D. (1990) Polymorphisms in the gene coding for cholesteryl ester transfer protein are related to plasma high-density lipoprotein cholesterol and transfer protein activity. *Clin. Sci.* 79, 575-581

Fukasawa, M., Arai, H., and Inoue, K. (1992) Establishment of anti-human cholesteryl ester transfer protein monoclonal antibodies and radioimmunoassaying of the level of cholesteryl ester transfer protein in human plasma. *J. Biochem.* 111, 696-698

Glomset, J.A. (1968) The plasma lecithin:cholesterol acyltransferase reaction. *J. Lipid Res.* 9, 155-167

Gordon, D.J., and Rifkind, B.M. (1989) High-density lipoprotein - the clinical implications of recent studies. *N. Engl. J. Med.* 321, 1311-1316

Gotoda, T, Kinoshita, M., Shimano, H., Harada, K., Shimada, M. *et al.* (1993) Cholesteryl ester transfer protein deficiency caused by a nonsense mutation detected in patient's macrophage mRNA. *Biochem. Biophys. Res. Commun.* 194, 519-524

Gotto Jr., A.M., LaRosa, J.C., Hunninghake, D., Grundy, S.M., Wilson, P.W., *et al.* (1990) The cholesterol facts. A summary of the evidence relating dietary fats, serum cholesterol, and coronary heart disease. *Circulation* 81, 1721-1733

Granot, E., Tabas, I., and Tall, A.R. (1987) Human cholesteryl ester transfer protein enhances the transfer of cholesteryl ester from high density liporpoteins into cultured Hep G2 cells. *J. Biol. Chem.* 262, 3482-3487

Green, S.R., and Pittman, R.C. (1991) Comparative acyl specificities for transfer and selective uptake of high density lipoprotein cholesteryl esters. *J. Lipid Res.* 32, 457-467

Green, S.R., Beltz, W.F., Goldberg, D.I., and Pittman, R.C. (1989) Cholesteryl oleyl and linoleyl ethers do not trace their ester counterparts in animals with cholesteryl ester transfer activity. *J. Lipid Res.* 30, 1405-1410

Groener, J.E.M., and Kostner, G.M. (1987) Lipid transfer protein-catalyzed exchange of cholesteryl ester between high density lipoproteins and apoB-containing lipoproteins. *J. Lipid Res.* 28, 1053-1056

Groener, J.E.M., van Rozen, A.J., and Erkelens, D.W. (1984) Cholesteryl ester transfer activity. Localization and role in distribution of cholesteryl ester among lipoproteins in man. *Atherosclerosis* 50, 2661-271

Groener, J.E.M., van Gent, T., and van Tol, A. (1989) Effect of lipid transfer protein on plasma lipids, apolipoproteins and metabolism of high-density lipoprotein cholesteryl ester in the rat. *Biochim. Biophys. Acta* 1002, 93-100

Guerin, M., Dolphin, P.J., Talussot, C., Gardette, J., Bertheze, F. *et al.* (1995) Pravastatin modulates cholesteryl ester transfer from HDL to apoB-containing lipoproteins and lipoprotein subspecies profile in familial hypercholesterolemia. *Arterioscl. Thromb. Vasc. Biol.* 15, 1359-1368

Guyard-Dangremont, V., Lagrost, L., and Gambert, P. (1994) Comparative effects of purified apolipoproteins A-I, A-II, and A-IV on cholesteryl ester transfer protein activity. *J. Lipid Res.* 35, 982-992

Ha, Y.C., and Barter, P.J. (1982) Differences in plasma cholesteryl ester transfer activity in sixteen vertebrate species. *Comp. Biochem. Physiol.* 71B, 265-269

Ha, Y.C., Calvert, G.D., McIntosh, G.H., and Barter, P.J. (1981) A physiologic role for the cholesterol ester transfer protein: *in vivo* studies in rabbits and pigs. *Metabolism* 30, 380-383

Ha, Y.C., Gorjatschko, L., and Barter, P.J. (1983) Changes in the density distribution of pig high density lipoproteins during incubation *in vitro*. Influence of esterified cholesterol transfer activity. *Atherosclerosis* 48, 253-263

Ha, Y.C., Chang, L.B.F., and Barter, P.J. (1985) Effects of injecting exogenous lipid transfer protein into rats. *Biochim. Biophys. Acta* 833, 203-210

Hannuksela, M., Marcel, Y.L., Kesaniemi, Y.A., and Savolainen, M.J. (1992) Reduction in the concentration and activity of plasma cholesteryl ester transfer protein by alcohol. *J. Lipid Res.* 33, 737-744

Hara, H., and Yokoyama, S. (1991) Interaction of free apolipoproteins with macrophages. Formation of high density lipoprotein-like lipoproteins and reduction of cellular cholesterol. *J. Biol. Chem.* 266, 3080-3086

Hayek, T., Chajek-Shaul, T., Walsh, A., Agellon, L.B., Moulin, P. *et al.* (1992) An interaction between the human cholesteryl ester transfer protein (CETP) and apolipoprotein A-I genes in transgenic mice results in profound CETP-mediated depression of high density lipoprotein cholesterol levels. *J. Clin. Invest.* 90, 505-510

Hayek, T., Azrolan, N., Verdery, R.B., Walsh, A. Chajek-Shaul, T. *et al.* (1993) Hypertriglyceridemia and cholesteryl ester transfer protein interact to dramatically alter high density lipoprotein levels, particle sizes, and metabolism. Studies in transgenic mice. *J. Clin. Invest.* 92, 1143-1152

Hayek, T., Masucci-Magoulas, L., Jiang, X., Walsh, A., Rubin, E. *et al.* (1995) Decreased early atherosclerotic lesions in hypertriglyceridemic mice expressing cholesteryl ester transfer protein transgene. *J. Clin. Invest.* 96, 2071-2074

Herz, J., and Willnow, T.E. (1995) Lipoprotein and receptor interactions *in vivo*. *Curr. Opin. Lipidol.* 6, 97-103

Hesler, C.B., Swenson, T.L., and Tall, A.R. (1987) Purification and characterization of a human plasma cholesteryl ester transfer protein. *J. Biol. Chem.* 262, 2275-2282

Hesler, C.B., Tall, A.R., Swenson, T.L., Weech, P.K., Marcel, Y.L. *et al.* (1988) Monoclonal antibodies to the M_r 74,000 cholesteryl ester transfer protein neutralize all of the cholesteryl ester and triglyceride transfer activities in human plasma. *J. Biol. Chem.* 263, 5020-5023

Hesler, C.B., Brown, M.L., Feuer, D.S., Marcel, Y.L., Milne, R.W. *et al.* (1989) Structure-function analysis of plasma cholesteryl ester transfer by protease digestion and expression of cDNA fragments in *Escherichia coli. J. Biol. Chem.* 264, 11317-11325

Hirano, K.-H., Matsuzawa, Y., Sakai, N., Hiraoka, H., Nozaki, S. (1992) Polydisperse low-density lipoproteins in hyperalphalipoproteinemic chronic alcohol drinkers in association with marked reduction of cholesteryl ester transfer protein activity. *Metabolism* 41, 1313-1318

Hopkins, G.J., and Barter, P.J. (1980) Transfers of esterified cholesterol and triglyceride between high density and very low density lipoproteins: *in vitro* studies of rabbits and humans. *Metabolism* 29, 546-550

Hopkins, G.J., and Barter, P.J. (1982) Dissociation of the *in vitro* transfers of esterified cholesterol and triglyceride between human lipoproteins. *Metabolism* 31, 78-81

Hopkins, G.J., Chang, L.B.F., and Barter, P.J. (1985) Role of lipid transfers in the formation of a subpopulation of small high density lipoproteins. *J. Lipid Res.* 26, 218-229

Horowitz, B.S., Goldberg, I.J., Merab, J., Vanni, T.M., and Ramakrishnan, R. *et al.* (1993) Increased plasma and renal clearance of an exchangeable pool of apolipoprotein A-I in subjects with low levels of high density lipoprotein cholesterol. *J. Clin. Invest.* 91, 1743-1752

Ierides, M., Dousset, N., Potier, M., Manent, J., Carton, M. *et al.* (1985) Cholesteryl ester transfer protein. Size of the functional unit determined by radiation inactivation. *FEBS Lett.* 193, 59-62

Ihm, J., Harmony, J.A.K., Ellsworth, J., and Jackson, R.L. (1980) Simultaneous transfer of cholesteryl ester and phospholipid by protein(s) isolated from human lipoprotein-free plasma. *Biochem. Biophys. Res. Commun.* 93, 1114-1120

Ihm, J., Ellsworth, J.L., Chataing, B., and Harmony, J.A.K. (1982a) Plasma protein-facilitated coupled exchange of phosphatidylcholine and cholesteryl ester in the absence of cholesterol esterification. *J. Biol. Chem.* 257, 4818-4827

Ihm, J., Quinn, D.M., Busch, S.J., Chataing, B., and Harmony, J.A.K. (1982b) Kinetics of plasma protein-catalyzed exchange of phosphatidylcholine and cholesteryl ester between plasma lipoproteins. *J. Lipid Res.*. 23, 1328-1341

Ikewaki, K., Rader, D.J., Sakamoto, T., Nishikawa, M., Wakimoto, N. *et al.* (1993) Delayed catabolism of high density lipoprotein apolipoproteins A-I and A-II in human cholesteryl ester transfer protein deficiency. *J. Clin. Invest.* 92, 1650-1658

Ikewaki, K., Nishiwaki, M., Sakamoto, T., Ishikawa, T., Fairwell, T. *et al.* (1995) Increased catabolic rate of low density lipoproteins in humans with cholesteryl ester transfer protein deficiency. *J. Clin. Invest.* 96, 1573-1581

Inazu, A., Brown, M.L., Hesler, C.B., Agellon, L.B., Koizumi, J. *et al.* (1990) Increased high density lipoprotein levels caused by a common cholesteryl-ester transfer protein gene mutation. *N. Engl. J. Med.* 323, 1234-1238

Inazu, A., Quinet, E.M., Wang, S., Brown, M.L., Stevenson, S. *et al.* (1992) Alternative splicing of the mRNA encoding the human cholesteryl ester transfer protein. *Biochemistry* 31, 2352-2358

Inazu, A., Jiang, X.-C., Haraki, T, Yagi, K, Kamon, N. (1994) Genetic cholesteryl ester transfer protein deficiency caused by two prevalent mutations as a major determinant of increased levels of high density lipoprotein cholesterol. *J. Clin. Invest.* 94, 1872-1882

Jarnagin, A.S., Kohr, W., and Fielding, C. (1987) Isolation and specificity of a M_r 74,000 cholesteryl ester transfer protein from human plasma. *Proc. Natl. Acad. Sci. USA* 84, 1854-1857

Jauhiainen, M., Metso, J., Pahlman, R., Blomqvist, S., van Tol, A. *et al.*, (1993) Human plasma phospholipid transfer protein causes high density lipoprotein conversion. *J. Biol. Chem.* 286, 4032-4036

Jiang, X.C., Moulin, P., Quinet, E., Goldberg, I.J., Yacoub, L.K. *et al.* (1991) Mammalian adipose tissue and muscle are major sources of lipid transfer protein mRNA. *J. Biol. Chem.* 266, 4631-4639

Jiang, X.C., Agellon, L.B., Walsh, A., Breslow, J.L., and Tall, A. (1992) Dietary cholesterol increases transcription of the human cholesteryl ester transfer protein gene in transgenic mice. Dependence on natural flanking sequences. *J. Clin. Invest.* 90, 1290-1295

Jiang, X.C., Masucci-Magoulas, L., Mar, J., Lin, M., Walsh, A. *et al.* (1993) Down-regulation of mRNA for the low density lipoprotein receptor in transgenic mice containing the gene for human cholesteryl ester transfer protein. Mechanism to explain accumulation of lipoprotein B particles. *J. Biol. Chem.* 268, 27406-27412

Jiao, S., Cole, T.G., Kitchens, R.T., Pfleger, B., and Schonfeld, G. (1990) Genetic heterogeneity of lipoproteins in inbred strains of mice: analysis by gel-permeation chromatography. *Metabolism* 39, 155-160

Johnson, W.J., Mahlberg, F.H., Rothblat, G.H., and Phillips, M.C. (1991) Cholesterol transport between cells and high-density lipoproteins. *Biochim. Biophys. Acta* 1085, 273-298

Kahri, J., Syvanne, M., and Taskinen, M.-R. (1994) Plasma cholesteryl ester transfer protein activity in non-insulin dependent diabetic patients with and without coronary artery disease. *Metabolism* 43, 1498-1502

Kane, J.P., and Havel., R.J. (1989) Disorders of the biogenesis and secretion of lipoproteins containing the B apolipoproteins. In *The metabolic basis of inherited disease* (Scriver, C.R., Beaudet, A.L., Sly, W.S., and Valle, D., eds) McGraw-Hill, New York, NY, 1139-1164

Kato, H., Nakanishi, T., Arai, H., Nishida, H.I., and Hishida, T. (1989) Purification, microheterogeneity,, and stability of human lipid transfer protein. *J. Biol. Chem.* 264, 4082-4087

Khoo, J.C., Miller, E., McLoughlin, P., and Steinberg, D. (1990) Prevention of low density lipoprotein aggregation by high density lipoprotein or apolipoprotein A-I. *J. Lipid Res.* 31, 645-652

Kinoshita, M., Arai, H., Fukasawa, M., Watanabe, T., Tsukamoto, K. *et al.* (1993) Apolipoprotein E enhances lipid exchange between lipoproteins mediated by cholesteryl ester transfer protein. *J. Lipid Res.* 34, 261-268

Ko, K.W.S., Oikawa, K., Ohnishi, T., Kay, C.M., and Yokoyama, S. (1993) Purification, characterization, and conformational analysis of rabbit plasma lipid transfer protein. *Biochemistry* 32, 6729-6736

Koizumi, J., Inazu, A., Yagi, K., Koizumi, I., Uno, Y. *et al.* (1991) Serum lipoprotein lipid concentration and composition in homozygous and heterozygous patients with cholesteryl ester transfer protein deficiency. *Atherosclerosis* 90, 189-196

Kondo, I., Berg, K., Drayna, D., and Lawn, R. (1989) DNA polymorphism at the locus for cholesteryl ester transfer protein (CETP) is associated with high density lipoprotein cholesterol and apolipoprotein levels. *Clin. Genet.* 35, 49-56

Kotake, H., Li, Q., Ohnishi, T., Ko, K.W.S., Agellon, L.B., and Yokoyama, S. (1996) Expression and secretion of rabbit plasma cholesteryl ester transfer protein by *Pichia pastoris*. *J. Lipid Res.* 37, 599-605

Krieger, M. (1992) Molecular flypaper and atherosclerosis: structure of the macrophage scavenger receptor. *Trends Biochem. Sci.* 17, 141-146

Kunitake, S.T., Jarvis, M.R., Hamilton, R.L., and Kane, J.P. (1992) Binding of transition metals by apolipoprotein A-I-containing plasma lipoproteins: inhibition of oxidation of low density lipoproteins. *Proc. Natl. Acad. Sci. USA* 89, 6993-6997

Kunitake, S.T., Mendel, C.M., and Hennessy, L.K. (1992b) Interconversion between apolipoprotein A-I-containing lipoproteins of pre-beta and alpha electrophoretic mobilities. *J. Lipid Res.* 33, 1807-1816

Kushwaha, R.S., Rainwater, D.L., Williams, M.C., Getz, G.S., and McGill Jr, M.C. (1990) Impaired plasma cholesteryl ester transfer with accumulation of larger high density lipoproteins in some families of baboons (*Papio* sp.). *J. Lipid Res.* 31, 965-973

Kushwaha, R.S., Hasan, S.Q., McGill Jr., H.C., Getz, G.S., Dunham, R.G. *et al.* (1993) Characterization of cholesteryl ester transfer protein inhibitor from plasma of baboons (*Papio* sp.). *J. Lipid Res.* 34, 1285-1297

Lagrost, L. (1994) Regulation of cholesteryl ester transfer protein (CETP) activity: review of *in vitro* and *in vivo* studies. *Biochim. Biophys. Acta* 1215, 209-236

Lagrost, L, and Barter, P.J. (1991) Effects of various non-esterified fatty acids on the particle size redistribution of high density lipoproteins induced by the human cholesteryl ester transfer protein. *Biochim. Biophys. Acta* 1082, 204-210

Lagrost, L., Gambert, P., Dangremont, V., Athias, A., and Lallemant, C. (1990) Role of cholesteryl ester transfer protein (CETP) in the HDL conversion process as evidenced by using anti-CETP monoclonal antibodies. *J. Lipid Res.* 31, 1569-1575

Lusis, A.J., Zollman, S., Sparkes, R.S., Kliasak, I., Mohandas, T. *et al.* (1987) Assignment of the human gene for cholesteryl ester transfer protein to chromosome 16q12-16q21. *Genomics* 1, 232-235

Mackness, M.I., and Durrington, P.N. (1992) Lipoprotein separation and analysis for clinical studies. In *Lipoprotein analysis. A practical approach* (Converse, C.A., and Skinner, E.R., eds) IRL Press, Oxford, Great Britain, 1-42

Mann, C.J., Yen, F.T., Grant, A.M., and Bihain, B.E. (1991) Mechanism of plasma cholesteryl ester transfer in hypertriglyceridemia. *J. Clin. Invest.* 88, 2059-2066

Marcel, Y.L., Vezina, C., Teng, B., and Sniderman, A. (1980) Transfer of cholesterol esters between human high density lipoproteins and triglyceride-rich lipoproteins controlled by a plasma protein factor. *Atherosclerosis* 35, 127-133

Marcel, Y.L., McPherson, R., Hogue, M., Czarnecka, H., Zawadski, Z. (1990) Distribution and concentration of cholesteryl ester transfer protein in plasma of normolipidemic subjects. *J. Clin. Invest.* 85, 10-17

Marotti, K.R., Castle, C.K., Murray, R.W., Rehberg, E.F., Polites, H.G. *et al* (1992) The role of cholesteryl ester transfer protein in primate apolipoprotein A-I metabolism. Insights from studies with transgenic mice. *Arterioscler. Thromb.* 12, 736-744

Marotti, K.R., Castle, C.K., Boyle, T.P., Lin, A.H., Murray, R.W. *et al.* (1993) Severe atherosclerosis in transgenic mice expressing simian cholesteryl ester transfer protein. *Nature* 364, 73-75

Martin, L.J., Conelly, P.W., Nancoo, D., Wood, N., Zhang, Z.J. *et al.* (1993) Cholesteryl ester transfer protein and high density lipoprotein responses to cholesterol feeding in men: relationship to apolipoprotein E genotype. *J. Lipid Res.* 34, 437-446

Masucci-Magoulas, L., Moulin, P., Jiang, X.C., Richardson, H., Walsh, A. *et al.* (1995) Decreased cholesteryl ester transfer protein (CETP) mRNA and protein and increased high density lipoprotein following lipopolysaccharide administration in human CETP transgenic mice. *J. Clin. Invest.* 95, 1587-1594

McPherson, R. (1993) Function of cholesteryl ester transfer protein in reverse cholesterol transport. *Can. Med. Assoc. J.* 148, 2165-21266

McPherson, R., Hogue, M., Milne, R.W., Tall, A.R., and Marcel, Y.L. (1991a) Increase in plasma cholesteryl ester transfer protein during probucol treatment. Relation to changes in high density lipoprotein composition. *Arterioscl. Thromb.* 11, 476-481

McPherson, R., Mann, C.J., Tall, A.R., Hogue, M., Martin, L. *et al.* (1991b) Plasma concentrations of cholesteryl ester transfer protein in hyperlipoproteinemia. Relation to cholesteryl ester transfer protein activity and other variables. *Arterioscl. Thromb.* 11, 797-804

Melchior, G.W., Castle, C.K., Murray, R.W., Blake, W.L., Dinh, D.M. *et al.* (1994) Apolipoprotein A-I metabolism in cholesteryl ester transfer protein transgenic mice. Insights into the mechanisms responsible for low plasma high density lipoprotein levels. *J. Biol. Chem.* 269, 8044-8051

Melchior, G.W., Greelee, K.A., Castle, C.K., Prough, M.J., Milne, R.W. *et al.* (1995) Evidence that cynomolgus monkey cholesteryl ester transfer protein has two neutral lipid binding sites. *J. Biol. Chem.* 270, 21068-21074

Miller, N.E. (1990) HDL metabolism and its role in lipid transport. *Eur. Heart J.* 11 (Suppl. H) 1-3

Milner, T.G., Ko, K.W.S., Ohnishi, T., and Yokoyama, S. (1991) Enhancement of human lipid transfer protein reaction by apolipoprotein. *Biochim. Biophys. Acta* 1082, 71-78

Morton, R.E. (1985) Binding of plasma-derived lipid transfer protein to lipoprotein substrates. The role of binding in the lipid transfer process. *J. Biol. Chem.* 260, 12593-12599

Morton, R.E. (1986) Specificity of lipid transfer protein for molecular species of cholesteryl ester. *J. Lipid Res.* 27, 523-529

Morton, R.E. (1988a) Interaction of plasma-derived lipid transfer protein with macrophages in culture. *J. Lipid Res.* 29, 1367-1377

Morton, R.E. (1988b) Free cholesterol is a potent regulator of lipid transfer protein function. *J. Biol. Chem.* 263, 12235-12241

Morton, R.E. (1990) Interaction of lipid transfer protein with plasma lipoproteins and cell membranes. *Experientia* 46, 552-559

Morton, R.E., and Green, D.J. (1994) Regulation of lipid transfer between lipoproteins by an endogenous plasma protein: selective inhibition among lipoprotein classes. *J. Lipid Res.* 35, 836-847

Morton, R.E., and Steinbrunner, J.V. (1990) Concentration of neutral lipids in the phospholipid surface of substrate particles determines lipid transfer protein activity. *J. Lipid Res.* 31, 1559-1567

Morton, R.E., and Zilversmit, D.B. (1981a) The separation of apolipoprotein D from cholesteryl ester transfer protein. *Biochim. Biophys. Acta* 663, 350-355

Morton, R.E., and Zilversmit, D.B. (1981b) A plasma inhibitor of triglyceride and cholesteryl ester transfer activities. *J. Biol. Chem.* 256, 11992-11995

Morton, R.E., and Zilversmit, D.B. (1982) Purification and characterization of lipid transfer protein(s) from human lipoprotein-deficient plasma. *J. Lipid Res.* 23, 1058-1067

Morton, R.E., and Zilversmit, D.B. (1983) Inter-relationship of lipids transferred by the lipid-transfer protein isolated from human lipoprotein-deficient plasma. *J. Biol. Chem.* 258, 11751-11757

Moulin, P., Appel, G.B., Ginsberg, H.N., and Tall, A.R. (1992) Increased concentration of plasma cholesteryl ester transfer protein in nephrotic syndrome: role in dyslipidemia. *J. Lipid Res.* 33, 1817-1822

Moulin, P., Cheung, M.C., Bruce, C., Zhong, S., Cocke, T. *et al.* (1994) Gender effects on the distribution of the cholesteryl ester transfer protein in apolipoprotein A-I-defined lipoprotein subpopulations. *J. Lipid Res.* 35, 793-802

Muzya, G.I., Korobkova, E.N., Golovanova, N.K., and Bergelson, L.D. (1987) Influence of prostaglandins on the lipid transfer between human high and low density lipoproteins. *FEBS Lett.* 220, 371-375

Nagashima, M., McLean, J.W., and Lawn, R.M. (1988) Cloning and mRNA tissue distribution of rabbit cholesteryl ester transfer protein. *J. Lipid Res.* 29, 1643-1649

Nestel, P.J., Reardon, M., and Billington, T. (1979) *In vivo* transfer of cholesteryl esters from high density lipoproteins to very low density lipoproteins in man. *Biochim. Biophys. Acta* 573, 403-407

Newnham, H.H., and Barter, P.J. (1990) Synergistic effects of lipid transfers and hepatic lipase in the formation of very small high-density lipoproteins during incubation of human plasma. *Biochim. Biophys. Acta* 1044, 57-64

Ng, D.S., Leiter, L.A., Vezina, C., Conelly, P.W., and Hegele, R.A. (1994) Apolipoprotein A-I Q[-2]X causing isolated apolipoprotein A-I deficiency in a family with analphalipoproteinemia. J. Clin. Invest. 93, 223-229

Nichols, A.V., and Smith, L. (1965) Effect of very low-density lipoproteins on lipid transfer in incubated serum. *J. Lipid Res.* 6, 206-210

Nishida, H.I., Kato, H., and Nishida, T. (1990) Affinity of lipid transfer protein for lipid and lipoprotein particles as influenced by lecithin-cholesterol acyltransferase. *J. Biol. Chem.* 265, 4876-4883

Nishida, H.I., Arai, H., and Nishida, T. (1993) Cholesteryl ester transfer mediated by lipid transfer protein as influences by changes in the charge characteristics of plasma lipoproteins. *J. Biol. Chem.* 268, 16352-16360

Nishide, T., Tollefson, J.H., and Albers, J.J. (1989) Inhibition of lipid transfer by a unique high density lipoprotein subclass containing an inhibitor protein. *J. Lipid Res.* 30, 149-158

Ogawa, Y., and Fielding, C.J. (1985) Assay of cholesteryl ester transfer activity and purification of a cholesteryl ester transfer protein. *Meth. Enzymol.* 111, 274-285

Ohnishi, T., and Yokoyama, S. (1993) Activation of human lipid transfer protein by apolipoproteins. *Biochemistry* 32, 5029-5035

Ohnishi, T., Yokoyama, S., and Yamamoto, A. (1990) Rapid purification of human plasma lipid transfer protein. *J. Lipid Res.* 31, 397-406

Ohnishi, T., Hicks, L.D., Oikawa, K., Kay. C.M., and Yokoyama, S. (1994a) Properties of human plasma lipid transfer protein in aqueous solution and at interfaces. *Biochemistry* 33, 6093-6099

Ohnishi, T., Tan, C., and Yokoyama, S. (1994b) Selective transfer of cholesteryl ester over triglyceride by human plasma lipid transfer protein between apolipoprotein-activated lipid microemulsions. *Biochemistry* 33, 4533-4542

Ohta, T., Nakamura, R., Takata, K., Saito, Y., Yamashita, S. *et al.* (1995) Structural and functional differences of subspecies of apoA-I-containing lipoprotein in patients with plasma cholesteryl ester transfer protein deficiency. *J. Lipid Res..* 36, 696-704

Oschry, Y., and Eisenberg, S. (1982) Rat plasma lipoproteins: re-evaluation of a lipoprotein system in an animal devoid of cholesteryl ester transfer activity. *J. Lipid Res.* 23, 1099-1106

Pape, ME, Rehberg, EF, Marotti, KR, and Melchior, GW (1991a) Molecular cloning, sequence, and expression of cynomolgus monkey cholesteryl ester transfer protein. *Arterioscler. Thromb.* 11, 1759-1771

Pape, M.E., Ulrich, R.G., Rea, T.J., Marotti, K.R., and Melchior, G.W. (1991b) Evidence that the nonparenchymal cells of the liver are the principal source of cholesteryl ester transfer protein in primates. *J. Biol. Chem.* 266, 12829-12831

Parthasarathy, S., Barnett, J., and Fong, L.G. (1990) High-density lipoprotein inhibits the oxidative modification of low-density lipoprotein. *Biochim. Biophys. Acta* 1044, 275-283

Pattnaik, N.M., and Zilversmit, D.B. (1979) Interaction of cholesteryl ester exchange protein with human plasma lipoproteins and phospholipid vesicles. *J. Biol. Chem.* 254, 2782-2786

Pattnaik, N.K., Montes, A., Hughes, B., and Zilversmit, D.B. (1978) Cholesteryl ester exchange protein in human plasma. Isolation and characterization. *Biochim. Biophys. Acta* 530, 428-438

Pieters, M.N., Schouten, D., and Van Berkel, T.J.C. (1994) *In vitro* and *in vivo* evidence for the role of HDL in reverse cholesterol transport. *Biochim. Biophys. Acta* 1225, 125-134

Pownall, H.J., and Gotto Jr., A.M. (1992) Human plasma apolipoproteins in biology and medicine. In *Structure and function of apolipoproteins* (Rosseneu, M., ed.) CRC Press, Boca Raton, FL, 1-32

Quig, D.W., and Zilversmit, D.B. (1986) Disappearance and effects of exogenous lipid transfer activity in rats. *Biochim. Biophys. Acta* 879, 171-178

Quig, D.W., Arbeeny, C.M., and Zilversmit, D.B. (1991) Effects of hyperlipidemias in hamsters on lipid transfer protein activity and unidirectional cholesteryl ester transfer in plasma. *Biochim. Biophys. Acta* 1083, 257-264

Quinet, E.M., Agellon, L.B., Kroon, P.A., Marcel, Y.L., Lee, Y.-C. *et al.* (1990) Atherogenic diet increases cholesteryl ester transfer protein messenger RNA levels in rabbit liver. *J. Clin. Invest.* 85, 357-363

Quinet, E., Tall, A., Ramakrishnan, R., and Rudel, L. (1991) Plasma lipid transfer protein as a determinant of the atherogenicity of monkey plasma lipoproteins. *J. Clin. Invest.* 87, 1559-1566

Quinet, E.M., Huerta, P., Nancoo, D., Tall, A.R., Marcel, Y.L. *et al.* (1993a) Adipose tissue cholesteryl ester transfer protein mRNA in response to probucol treatment: cholesterol and species dependence. *J. Lipid Res.* 34, 845-852

Quinet, E., Yang, T.P., Marinos, C., and Tall, A. (1993b) Inhibition of the cellular secretion of cholesteryl ester transfer protein by a variant protein formed by alternative splicing of mRNA. *J. Biol. Chem.* 268, 16891-16894

Rajaram, O.V., White, G.H., and Barter, P.J. (1980) Partial purification and characterization of a triacylglycerol-transfer protein from rabbit serum. *Biochim. Biophys. Acta* 617, 383-392

Rajaram, O.V., Chan, R.Y.S., and Sawyer, W.H. (1994) Effect of unesterified cholesterol on the activity of cholesteryl ester transfer protein. *Biochem. J.* 304, 423-430

Ravnik, S.E., Zarutskie, P.W., and Muller, C.H. (1992) Purification and characterization of a human follicular fluid lipid transfer protein that stimulates human sperm capacitation. *Biol. Reprod.* 47, 1126-1133

Rinninger, F., and Pittman, R.C. (1989) Mechanism of the cholesteryl ester transfer protein-mediated uptake of high density lipoprotein cholesteryl esters by Hep G2 cells. *J. Biol. Chem.* 264, 6111-6118

Rinninger, F., Deichen, J.T., Jackle, S., Windler, E., and Greten, H. (1994) Selective uptake of high-density lipoprotein-associated cholesteryl esters and high-density lipoprotein particle uptake by human monocyte-macrophages. *Atherosclerosis* 105, 145-157

Ross, R. (1993) The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature* 362, 801-809

Rye, K.-A., Garrety, K.H., and Barter, P.J. (1992) Changes in the size of reconstituted high density lipoproteins during incubation with cholesteryl ester transfer protein: the role of apolipoproteins. *J. Lipid Res.* 33, 215-224

Sakai, N., Matsuzawa, Y., Hirano, K.-I., Yamashita, S., Nozaki, S. *et al.* (1991) Detection of two species of low density lipoprotein particles in cholesteryl ester transfer protein deficiency. *Arterioscler. Thromb.* 11, 71-79

Sammett, D., and Tall, A. R. (1985) Mechanisms of enhancement of cholesteryl ester transfer protein activity by lipoplysis. *J. Biol. Chem.* 260, 6687-6697

Savolainen, M.J., Hannuksela, M., Seppanen, S., Kervinin, K., and Kesaniemi, Y.A. (1990) Increased high-density lipoprotein cholesterol concentration in alcoholics is related to low cholesteryl ester transfer protein activity. Eur. *J. Clin. Invest.* 20, 593-599

Seip, R.L., Moulin, P., Cocke, T., Tall, A., Kohrt, W.M. *et al.* (1993) Exercise training decreases plasma cholesteryl ester transfer protein. *Arterioscler. Thromb.* 13, 1359-1367

Serrat-Serrat, J., Ordonez-Llanos, J., Serra-Grima, R., Gomez-Gerique, J.A., Pellicer-Thoma, E. *et al.* (1993) Marathon runners presented lower serum cholesteryl ester transfer activity than sedentary subjects. *Atherosclerosis* 101, 43-49

Shen, B.W., Scanu, A.M., and Kezdy, F.J. (1977) Structure of human serum lipoproteins inferred from compositional analysis. *Proc. Natl. Acad. Sci. USA* 74, 837-841

Sniderman, A., Teng, B., Vezina, C., and Marcel, Y.L. (1978) Cholesterol ester exchange between human plasma high and low density lipoproteins mediated by a plasma protein factor. *Atherosclerosis* 31, 327-333

Son, Y.-S.C., and Zilversmit, D.B. (1984) Purification and characterization of human plasma proteins that inhibit lipid transfer activities. *Biochim. Biophys. Acta* 795, 473-480

Son, Y.-S.C., and Zilversmit, D.B. (1986) Increased lipid transfer activities in hyperlipidemic rabbit plasma. *Arterioscler.* 6, 345-351

Sparks, D., Frohlich, J.J., and Pritchard, P.H. (1991) Lipid transfer proteins, hypertriglyceridemia, and reduced high-density lipoprotein cholesterol. *Am. Heart J.* 122, 601-607

Sperker, B., Mark, M., and Budzinski, R.-M. (1993) The expression of human plasma cholesteryl-ester-transfer protein in HepG2 cells is induced by sodium butyrate. Quantification of low mRNA levels by polymerase chain reaction. *Eur. J. Biochem.* 218, 945-950

Stein, Y., Stein, O., Olivecrona, T., and Halperin, G. (1985) Putative role of cholesteryl ester transfer protein in removal of cholesteryl ester from vascular interstitium, studied in a model system in cell culture. Biochim. Biophys. Acta 834, 336-345

Stein, O., Halperin, G., and Stein, Y. (1986) Cholesteryl ester efflux from extracellular and cellular elements of the arterial wall. Model systems in culture with cholesteryl linoleyl ether. *Arterioscler..* 6, 70-78

Stein, O., Hollander, G., Dabach, Y., Halperin, G., and Stein, Y. (1989) Use of ^{3}H-cholesteryl linoleyl ether as a quantitative marker for loss of cholesteryl ester during regression of cholesterol-induced aortic atheromas in rabbits. *Arterioscl.* 9, 247-252

Stein, Y., Dabach, Y., Hollander, G., and Stein, O. (1990) Cholesteryl ester transfer activity in hamster plasma: increase by fat and cholesterol rich diets. *Biochim. Biophys. Acta* 1042, 138-141

Stein, O., Dabach, Y., Hollander, G., and Stein, Y. (1991) Removal of cholesteryl ester from hepatic reticuloendothelial cells *in vivo* is not enhanced by cholesteryl ester transfer protein. *Biochim. Biophys. Acta* 1081, 238-240

Steinberg, D., Parthasarathy, S., Carew, T.E., Khoo, J.C. and Witztum, J.L. (1989) Beyond cholesterol. Modifications of low-density lipoprotein that increase its atherogenicity. *N. Engl. J. Med.* 320, 915-924

Stevenson, S.C., Wang, S., Deng, L., and Tall, A.R. (1993) Human plasma cholesteryl ester transfer protein consists of a mixture of two forms reflecting variable glycosylation at asparagine 341. *Biochemistry* 32, 5121-5126

Subbaiah, P.V., and Rodby, R.A. (1994) Abnormal acyltransferase activities and accelerated cholesteryl ester transfer in patients with nephrotic syndrome. *Metabolism* 43, 1126-1133

Swenson, T.L., Simmons, J.S., Hesler, C.B., Bisgaier, C., and Tall, A.R. (1987) Cholesteryl ester transfer protein is secreted by HepG2 cells and contains asparagine-linked carbohydrate and sialic acid. *J. Biol. Chem.* 262, 16271-16274

Swenson, T.L., Brocia, R.W., and Tall, A.R. (1988) Plasma cholesteryl ester transfer protein has binding sites for neutral lipids and phospholipids. *J. Biol. Chem.* 263, 5150-5157

Swenson, T.L., Hesler, C.B., Brown, M.L., Quinet, E., Trotta, P.P. *et al.* (1989) Mechanism of cholesteryl ester transfer protein inhibition by a neutralizing monoclonal antibody and mapping of the monoclonal antibody epitope. *J. Biol. Chem.* 264, 14318-14326

Takahashi, K., Jiang, X.-C., Sakai, N., Yamashita, S., Hirano, K. *et al.* (1993) A missense mutation in the cholesteryl ester transfer protein gene with possible dominant effects on plasma high density lipoproteins. *J. Clin. Invest.* 92, 2060-2064

Takahashi, F., Hasumi, K., and Endo, A. (1995) Modulation of plasma cholesteryl ester transfer by stachybotramide. *Biochim. Biophys. Acta* 1258, 70-74

Tall, A. (1995) Plasma lipid transfer proteins. *Annu. Rev. Biochem.* 64, 235-257

Tall, A.R. (1986) Plasma lipid transfer proteins. *J. Lipid Res.* 27, 361-367

Tall, A.R. (1990) Plasma high density lipoproteins. Metabolism and relationship to atherosclerosis. *J. Clin. Invest.* 86, 379-384

Tall, A.R. (1993) Plasma cholesteryl ester transfer protein. *J. Lipid Res.* 34, 1255-1274

Tall, A.R., Abreu, E., and Shuman, J. (1983) Separation of a plasma phospholipid transfer protein from cholesteryl ester/phospholipid exchange protein. *J. Biol. Chem.* 258, 2174-2180

Tall, A.R., Sammett, D., Viat, G.M., Deckelbaum, R., and Olivecrona, T. (1984) Lipoprotein lipase enhances the cholesteryl ester transfer protein-mediated transfer of cholesteryl esters from high density lipoproteins to very low density lipoproteins. *J. Biol. Chem.* 259, 9587-9594

Tall, A.R., Sammett, D., and Granot, E. (1986) Mechanisms of enhanced cholesteryl ester transfer from high density lipoproteins to apolipoprotein B-containing lipoproteins during alimentary lipemia. *J. Clin. Invest.* 77, 1163-1172

Tall, A.R., Granot, E., Brocia, R., Tabas, I., Hesler, C. *et al.* (1987) Accelerated transfer of cholesteryl esters in dyslipidemic plasma. Role of cholesteryl ester transfer protein. *J. Clin. Invest.* 79, 1217-1225

Tollefson, J.H., Ravnik, S., and Albers, J.J. (1988a) Isolation and characterization of a phospholipid transfer protein (LTP-II) from human plasma. *J. Lipid Res.* 29, 1593-1602

Tollefson, J.H., Liu, A., and Albers, J.J. (1988b) Regulation of plasma lipid transfer by the high-density lipoproteins. *Am. J. Physiol.* 255, E894-E902

Tu, A.-Y., Nishida, H.I., and Nishida, T. (1993) High density lipoprotein conversion mediated by a human plasma phospholipid transfer protein. *J. Biol. Chem.* 268, 23098-23105

Wang, S., Deng, L., Brown, M.L., Agellon, L.B., and Tall, A.R. (1991) Structure-function studies of human cholesteryl ester transfer protein by linker insertion scanning mutagenesis. Biochemistry 30, 3484-3490

Wang, S., Deng, L., Milne, R.W., and Tall, A.R. (1992) Identification of a sequence within the C-terminal 26 amino acids of cholesteryl ester transfer protein responsible for binding a neutralizing monoclonal antibody and necessary for neutral lipid transfer activity. *J. Biol. Chem.* 267, 17487-17490

Wang, S., Wang, X., Deng, L., Rassart, E., Milne, R.W. *et al.* (1993) Point mutagenesis of carboxyl-terminal amino acids of cholesteryl ester transfer protein. Opposite sides of an amphipathic helix important for cholesteryl ester transfer or for binding neutralizing antibody. *J. Biol. Chem.* 268, 1955-1959

Wang, S., Kussie, P., Deng, L., and Tall, A. (1995) Defective binding of neutral lipids by a carboxyl-terminal deletion mutant of cholesteryl ester transfer protein. Evidence for a carboxyl-terminal cholesteryl ester binding site essential for neutral lipid transfer activity. *J. Biol. Chem.* 270, 612-618

Warren, R.J., Ebert, D.L., Barter, P.J., and Mitchell, A. (1991) The regulation of hepatic lipase and cholesteryl ester transfer protein activity in the cholesterol fed rabbit. *Biochim. Biophys. Acta* 1086, 354-358

Weinberg, R.B., Cook, V.R., Jones, J.J., Kussie, P., and Tall, A.R. (1994) Interfacial properties of recombinant human cholesterol ester transfer protein. *J. Biol. Chem.* 269, 29588-29591

Whitlock, M.E., Swenson, T.L., Ramakrishnan, R., Leonard, M.T., Marcel, Y.L. *et al.* (1989) Monoclonal antibody inhibition of cholesteryl ester transfer protein activity in the rabbit. Effects on lipoprotein composition and high density lipoprotein cholesteryl ester metabolism. *J. Clin. Invest.* 84, 129-137

Williams, K.J., and Tabas, I. (1995) The response-to-retention hypothesis of early atherogenesis. *Arterioscl. Thromb. Vasc. Biol.* 15, 551-561

Yamashita, S., Sprecher, D.L., Sakai, N., Matsuzawa, Y., Tarui, S *et al.* (1990) Accumulation of apolipoprotein E-rich high density lipoproteins in hyperalphalipoprotienemic human subjects with plasma cholesteryl ester transfer protein deficiency. *J. Clin. Invest.* 86, 688-695

Yamashita, S., Hui, D.Y., Wetterau, J.R., Sprecher, D.L., Harmony, J.A.K. (1991) Characteristics of plasma lipoproteins in patients heterozygous for human plasma cholesteryl ester transfer protein (CETP) deficiency: plasma CETP regulates high density lipoprotein concentration and composition. *Metabolism* 40, 756-762

Yen, F.T., Deckelbaum, R.J., Mann, C.J., Marcel, Y.L., Milne, R.W. *et al.* (1989) Inhibition of cholesteryl ester transfer protein activity by monoclonal antibody. Effects on cholesteryl ester formation and neutral lipid mass transfer in human plasma. *J. Clin. Invest.* 83, 2018-2024

Young, P.M., and Brecher, P. (1981) Cholesteryl ester transfer from phospholipid vesicles to human high density lipoproteins. *J. Lipid Res.* 22, 944-954

Zawadski, Z., Milne, R.W., and Marcel, Y.L. (1991) Cu^{2+}-mediated oxidation of dialyzed plasma: effects on low and high density lipoproteins and cholesteryl ester transfer protein. *J. Lipid Res.* 32, 243-250

Zhong, S., Goldberg, I.J., Bruce, C., Rubin, E., Breslow, J. (1994) Human apoA-II inhibits the hydrolysis of HDL triglyceride and the decrease of HDL size induced by hypertriglyceridemia and cholesteryl ester transfer protein in transgenic mice. *J. Clin. Invest.* 94, 2457-2467

Zilversmit, D.B., Hughes, L.B., and Balmer, J. (1975) Stimulation of cholesterol ester exchange by lipoprotein-free rabbit plasma. *Biochim. Biophys. Acta* 409, 393-398

Zilversmit, D.B., Morton, R.E., Hughes, L.B., and Thompson, K.H. (1982) Exchange of retinyl and cholesteryl esters between lipoproteins of rabbit plasma. *Biochim. Biophys. Acta* 712, 88-93

CHAPTER 2

Fluorescence Assay for Measuring LTP Activity Used to Study Activation of LTP by Apolipoproteins[1]

2.1 Introduction

LTP facilitates the exchange and net transfer of neutral lipids between plasma lipoproteins. This activity provides the basis for physiological roles of LTP in modulation of HDL cholesterol levels, RCT, and HDL subclass interconversion. Thus, LTP activity is important in human plasma lipid transport, as well as having various implications towards atherosclerosis. Investigations into the regulation of LTP activity by plasma proteins have led to the characterization of proteins inhibitory to LTP (Son and Zilversmit, 1984; Nishide *et al.*, 1989; Kushwaha *et al.*, 1993), and of interactions between LTP and the enzyme LCAT (Nishida *et al.*, 1990).

Apolipoproteins are lipophilic polypeptides associated with the plasma lipoproteins. Not all the physiological roles of the major apolipoproteins are known. ApoA-I and A-II are the major HDL apolipoproteins and apoA-I is a co-factor for LCAT (Aron *et al.*, 1978) while apoA-II may inhibit hepatic lipase (Thuren *et al.*, 1991). ApoB is involved in intracellular assembly of TG-rich lipoproteins (Yao and McLeod, 1994) as well as being a ligand for the LDLR (Yang *et al.*, 1986). ApoC-II is a co-factor for lipoprotein lipase (Kinnunen *et al.*, 1977) and all C apolipoproteins inhibit remnant clearance by the liver (Windler and Havel, 1983). ApoE is a ligand for LDLR and LRP (Weisgraber, 1994). A role for exchangeable, amphiphilic apolipoproteins in stabilization of lipid microemulsion particles and stimulation of LTP mediated neutral lipid heteroexchange between these particles and LDL has been described (Nishikawa *et al.*, 1988). The specific effects of apolipoproteins on the lipid transfer process were unclear in that study since the lipoprotein pair studied in their assay system were heterogeneous in apolipoprotein and lipid composition.

[1] A version of this chapter has been published: Milner, T.G., Ko, K.W.S., Ohnishi, T., and Yokoyama, S. (1991) *Biochim. Biophys. Acta* 1082, 71-78. As second co-author of this paper, I contributed to the purification of the human LTP, discussion, and reading of the manuscript.

In the present study, the effects of apolipoproteins on LTP activity were characterized under more controlled conditions. An assay was developed using pyrene-labeled CE as a probe for monitoring neutral lipid transfer between artificially prepared lipid microemulsion particles. This allowed us to examine systematically the effects of exchangeable, amphiphilic apolipoproteins on LTP activity between lipoproteins almost homogeneous in lipid and protein composition.

2.2 Experimental Procedures

2.2.1 Preparation of Lipid Microemulsion Particles

Lipid microemulsion particles were prepared based on previously described methods (Okabe *et al.*, 1988). To prepare acceptor particles, 20 mg each of triolein (> 99% pure, Sigma) and egg PC (Avanti) were dried down and sonicated in 10 mL of 10 mM sodium phosphate (pH 7.4), 150 mM NaCl (Na-phosphate/NaCl buffer). Sonication was performed 15 times (70-100 watts) at 3 min intervals with 1 min breaks in between (Heat Systems) with the sample placed under a stream of nitrogen and cooled on ice. The mixture was then centrifuged at 99,000 rpm (404,000 x *g*) for 30 min (Beckman TLA-100.3) and the top layer (1.5 mL) of supernatant was applied onto a Sepharose CL-4B column (2.5 cm i.d. x 70 cm). The column was eluted with Na-phosphate/NaCl buffer, collecting 60 drop fractions and fraction 20 to 22 inclusive were pooled and used for experiments. To prepare donor particles, cholesterol-3-(6-(1-pyrene))hexanoate (Sigma) was premixed with the triolein and PC at ratios of from 0.2:5:5 to 1:5:5 (w/w/w) and microemulsion particles were prepared as above, except that the lipids were kept in darkness. Lipid concentrations were determined using enzymatic assay kits (Wako) to measure TG, choline-containing PL, and total cholesterol. The microemulsions were stored in darkness under argon at 4° C and used within 2 weeks.

2.2.2 Purification of LTP

LTP was partially purified from fresh human plasma obtained by plasmapheresis, according to the previously reported method (Ohnishi *et al.*, 1990). Briefly, lipoproteins were removed by precipitation with dextran-sulfate, followed by column chromatography of the lipoprotein-depleted plasma using Butyl-Toyopearl and CM-Toyopearl (both from Supelco), with LTP activity monitored by measuring the transfer of radiolabeled CE from LDL to HDL. The preparation obtained was approximately 20% pure LTP and most of the contaminants seen by SDS-PAGE (Laemmli, 1970) and Coomassie Blue staining were low M_r proteins. There were no bands corresponding to any apolipoproteins and the preparation was stored at -75° C in Na-phosphate/NaCl buffer.

2.2.3 *Purification of Apolipoproteins*

ApoA-I and A-II were isolated from fresh human plasma as described previously (Yokoyama *et al.*, 1982; Tajima *et al.*, 1983). Each protein exhibited a single band by SDS-PAGE (Laemmli, 1970). The proteins were lyophilized and stored at -75° C in argon and aqueous solutions of the apolipoproteins were prepared in Na-phosphate/NaCl buffer as previously described (Yokoyama *et al.*, 1982; Tajima *et al.*, 1983). Protein concentration of the solutions were calculated from their absorbance at 280 nm and the specific molar extinction coefficient for each respective apolipoprotein (Tajima *et al.*, 1983).

2.2.4 *Recording of Fluorescence Spectra*

Fluorescence spectra of microemulsion solutions were obtained at room temperature between emission wavelengths of 380 and 500 nm with an excitation wavelength of 320 nm (Roseman and Thompson, 1980; Massey *et al.*, 1982; Pownall and Smith, 1989) using a Hitachi F-2000 fluorescence spectrophotometer at maximum scanning speed (20 nm/s). Samples were kept under continuous flow of argon (10 mL/min) during measurement. The excitation lamp was kept off except during scans.

2.2.5 *Monitoring of Lipid Transfer*

Donor particles containing pyrene-labeled CE were mixed with acceptor particles at a 1:50 (w/w) ratio according to PC mass. Apolipoproteins, when used, were preincubated with each microemulsion separately, at the same weight ratio to PC, at room temperature for 30 min before mixing. Fluorescence scans were taken as described above. The ratio of the height of the peak at 470 nm to that at 395 nm was used as an indicator of pyrene concentration in the donor microemulsion particles. LTP was added to the mixture and lipid transfer was monitored by change of the fluorescence spectrum as a function of time. The final incubation volume was 1 mL and microemulsion concentration was 0.9 mM by PC unless otherwise specified.

2.3 Results

Fig. 2.1 shows a typical elution profile of donor lipid microemulsion preparation containing PC, TG and pyrene-labeled CE from a Sepharose CL-4B column after sonication and ultracentrifugation. The ratio of core TG to surface PC decreased as the size of the particle was reduced (Fig. 2.1, lower panel). The elution profile of cholesterol corresponded closely to that of TG, and the cholesterol/TG ratio indeed remained constant throughout the fractions (Fig. 2.1, upper panel), indicating homogeneous mixing of core lipids. Furthermore, the profile of the cholesterol/PC ratio was similar as that of the TG/PC ratio (not shown). Thus, pyrene-labeled CE is mainly associated with the TG core of the particle, as previously shown for incorporation of cholesteryl oleate into lipid microemulsion particles (Okabe *et al.*, 1988). Particles having a core to surface lipid ratio of approximately 1.2:1 by weight (fractions 20 to 22 inclusive), were pooled for use as donor particles in the experiments to follow.

A fluorescence spectrum of donor lipid microemulsion particles containing pyrene-labeled CE is shown in Figure 2.2. At an excitation wavelength of 320 nm, two emission peaks are defined at 395 nm and 470 nm, corresponding to monomeric and excimeric fluorescence of the pyrene moiety, respectively. Particles not containing pyrene-lipid showed negligible fluorescence. When the ratio of the excimeric peak to monomeric peak intensity (E_{470}/E_{395}, or f) was determined for microemulsions containing varying molar ratios of pyrene-labeled CE to core lipid, CE/(CE + TG), and when the former was plotted against the latter, a linear relationship was obtained (Fig. 2.3). Thus, the fluorescence ratio provides an indication of microscopic pyrene-CE concentration within the lipid microemulsion particle core.

To assay for lipid transfer, donor microemulsion particles containing pyrene-CE (approximately 2 mol% of core lipid) were combined with a 50-fold excess of acceptor particles, to give a final concentration of 0.9 mM by PC. When the E_{470}/E_{395} ratio was plotted as a function of time, a slow spontaneous decrease was observed in the absence of human LTP, which was only slightly enhanced in its presence at two concentrations (Fig. 2.4, top). The presence of bovine serum albumin (100 μg/mL) did not alter the transfer rate (not shown). Also, incubation of a high concentration of LTP (100 μg/mL) with pyrene-CE containing particles alone (0.1 mM by PC) did not result in change of fluorescence ratio for at least 30 min (not shown). When microemulsions were preincubated with apoA-I or A-II, 3.0 and 2.0 μM, respectively, sufficient to saturate their surface, the rate of decrease in E_{470}/E_{395} in the absence of LTP was not significantly different from spontaneous transfer (Fig. 2.4, middle and bottom). However, presence of either apolipoprotein markedly enhanced the transfer of pyrene-CE at each concentration of human LTP (Figure 2.4, middle and bottom).

In order to check whether fusion or aggregation, and not lipid transfer, might be responsible for the apparent decrease in core concentration of pyrene compound within the donor particles as indicated by decreasing fluorescence ratio, a reaction mixture of donor and acceptor particles was examined by gel permeation chromatography. A 1:4 mixture of donor and acceptor particles (final concentration 1 mM by PC) was passed through a Sepharose CL-4B column after 30 min incubation in the absence and presence of 100 μg/mL LTP and 3 μM apoA-I. Both samples eluted at the same position as the fractions originally pooled during preparation of the lipid microemulsion particles, tubes 20 to 22 (Fig. 2.5), indicating that changes in particle size due to fusion had not occurred under either condition.

The rate of lipid transfer was calculated by analysis of the data assuming a common rate constant k for both core lipids according to the equation $\ln (f - b) = -(2k/L_1)t + \ln (aC/L_1)$, where f is E_{470}/E_{395}, a and b are the slope and y-axis intercept of the plot in Figure 2.3, respectively, L_1 is the total core lipid concentration of donor particle, C is total pyrene-CE concentration in the incubation mixture (see Appendix). Thus, $\ln (E_{470}/E_{395} - 0.066)$ was plotted against time to yield the slope, $-2k/L_1$, using a least squares linear regression program for the initial 30% change of the $(f - b)$ value. The k/L_1 is then a rate constant of lipid transfer from the donor particle under the given experimental conditions.

The rate constants were determined for the two concentrations of LTP in the transfer assay. As shown in Figure 2.6, they were linearly related to the concentration of LTP. The maximal stimulation of pyrene-CE transfer between lipid microemulsions obtainable by LTP addition alone was not large, resulting in a mere doubling of the spontaneous transfer rate constant at the highest LTP concentration (100 µg/mL) used in our studies. The rate constant of lipid transfer in the presence of LTP was markedly increased, up to 7.5-fold, by either apoA-I or A-II when the surface of the lipid microemulsion particles were saturated with these apolipoproteins. Spontaneous transfer of core lipid was not significantly affected by the presence of apolipoproteins alone.

In order to approach the mechanism of the enhancement of LTP activity by apolipoproteins, the surface of the lipid microemulsion particles were partially saturated to various extents with apoA-I and the rate constants of lipid transfer were determined. The level of partial saturation of the surface was calculated using as the dissociation constant, $K_d = 1.6 \times 10^{-7}$ M, and the level of binding at saturation, $B_s = 2.5 \times 10^{-3}$ apoA-I/PL (mol/mol), determined previously for apoA-I (Tajima *et al.*, 1983), by the equation:

$$(\%\ \text{saturation}) = P_b/B_o = (P_t + K_d + B_o - (P_t + K_d + B_o)^2 - 4P_tB_o)\ /2B_o$$

where $B_o = B_s \times$ (PL concentration (M)) (since PL concentration was 1.39 mM, B_o was 3.48×10^{-6} M in this series of experiments) and P_b and P_t are total and bound apolipoprotein concentrations, respectively (Yokoyama 1990). As shown in Figure 2.7, the rate constant was linearly proportional to the extent of lipid microemulsion particle surface saturation by apoA-I.

2.4 Discussion

Pyrene compounds have been used for modeling spontaneous transfer of lipid between bilayer vesicles (Roseman and Thompson, 1980), as well as recombinant (Massey *et al*, 1982) and native (Charlton *et al.*, 1976) HDL. The principle behind the use of pyrene compounds to monitor lipid transfer is well established and has been adapted here to study protein mediated transfer of a core lipid analog, pyrene-CE (Fig. 2.8), between lipid microemulsion particles. The fluorescence emission spectrum of the pyrene moiety is concentration dependent both when pyrene compounds are dissolved in bulk solutions or when incorporated within microscopic hydrophobic environments such as detergent micelles (Pownall and Smith, 1973). At low concentrations of pyrene, the emissions peak at less than 400 nm originates from the excited singlet or monomeric state pyrene molecule. At higher concentrations, the emissions peak around 500 nm is from the excimeric state formed when an excited pyrene monomer and a ground state pyrene monomer transiently associate. Since excimer formation is a bimolecular process, the ratio of excimer to monomer emissions intensity is directly proportional to the pyrene concentration (Pownall and Smith, 1973 and Fig. 2.3), and decreases during net transfer of the probe from labeled donor to unlabeled acceptor particles. In this study, pyrene-CE was successfully incorporated into the core of lipid microemulsion particles to serve as a probe of neutral lipid transfer facilitated by LTP. Attributes of this system for investigating regulation of LTP activity are: (1) transfer can be monitored continuously since there is no need to separate donor and acceptor to obtain data; (2) donor and acceptor particles of very similar lipid composition are used, which is helpful for ruling out effects due to heterogeneity between lipoprotein pairs being studied; (3) the starting condition is lipid microemulsion particles alone, so effects of proteins or other factors on LTP activity can be readily assessed. Using this system we were able to show that LTP activity was enhanced by apoA-I and A-II in proportion to saturation of the lipid particle surface by these apolipoproteins.

Gel permeation chromatography was used to characterize the structural features of the lipid microemulsion particles as well as to verify the transfer process. Co-elution of pyrene-CE with TG during microemulsion preparation (Fig. 2.1) indicated that the pyrene compound was situated in the core of these particles previously characterized to be 26 nm in diameter, consisting of a neutral lipid core surrounded by a monolayer of PC and displaying saturable binding for exchangeable, amphiphilic apolipoproteins (Tajima *et al.*, 1983). Gel filtration chromatography also showed that the particle size was unchanged after incubation with LTP and apolipoproteins. Thus, the decreases in flourescence ratio during the assays in presence of LTP were due to an actual decrease of pyrene-CE microscopic concentration within the donor particles from transfer, and not fusion of donor and acceptor particles or net transfer of lipid from acceptor to donor particles, which would also lead to dilution of pyrene-CE; in these cases an increase in size of the pyrene-CE containing particles would be expected.

The fluorescence data were analyzed according to an equilibrium model of TG and pyrene-CE transfer between donor and acceptor lipid particles, in which case only exchange results (Pattnaik *et al.*, 1978; Barter and Jones, 1979). This form of analysis may be warranted because pyrene-CE is only a small percentage of the core lipid in the donor particles, so donor and acceptor particles are nearly homogeneous in lipid composition, and thus close to equilibrium. However, pyrene-CE transfer does involve heteroexchange and thus net transfer, especially during the initial portion of the assay, since acceptor particles lack CE. Furthermore, human LTP exhibits specificity for transfer of CE over TG (Morton and Zilversmit, 1983). Thus, the transfer rates obtained using pyrene-CE as a probe may not precisely represent CE exchange rates within an equilibrium model, but they should be a close approximation.

It is intriguing that pyrene-CE can serve as a transfer substrate for LTP. In addition to CE and TG, retinyl ester and cholesteryl ethers are transferred, albeit more slowly, by LTP (Morton and Zilversmit, 1982) and our lab has evidence that probucol incorporation into lipoproteins is enhanced by LTP (M. Tsujita and S. Yokoyama, personal communication). LTP prefers CE with lengthy and saturated acyl chains (Morton, 1986; Green and Pittman, 1991) and distinguishes between acyl and ether linkage of the acyl group, suggesting that the acyl group plays an important role in the interaction between LTP and neutral lipids. Our lab has recently shown that LTP appears to have strong selectivity for CE over TG during transfer between lipid microemulsion particles, much higher compared to transfer rates of CE versus TG between native lipoproteins (Ohnishi *et al.*, 1994). Epps *et al.* (1995) also reported large differences between LTP transfer rates for BODIPY-CE and -TG between synthetic lipid particles. Apparently, the additions of bulky moieties such as pyrene or BODIPY to CE do not preclude their transfer by LTP, although binding and transfer of neutral lipids to LTP can be inhibited by treatment of LTP with mAb (Swenson *et al.*, 1989) or chemical modification with pCMPS (Swenson *et al.*, 1988).

A role for exchangeable, amphiphilic apolipoproteins in stimulating LTP activity was demonstrated in this study. Apolipoproteins were previously found to stimulate LTP mediated neutral lipid heteroexchange between lipid microemulsion particles and LDL (Nishikawa *et al.*, 1988). However, under these conditions, apolipoproteins were also involved in stabilization of the microemulsions, and LDL contains free cholesterol as well as apoB integrated within its surface lipid, while the microemulsions contained neither. Such heterogeneities in lipid and protein composition between lipid donors and acceptors may have certain influences on lipid transfer, making it difficult to guage the effects of the added apolipoproteins on LTP activity. The present study showed that in an assay system consisting of nearly homogeneous donor and acceptor lipid microemulsion particles, the presence of surface bound apolipoproteins markedly enhances LTP activity. The results are more reliable in this study because fusion of microemulsion particles with LDL, a problem in the previous study, did not occur, and we found that both apoA-I and A-II could stimulate LTP activity up to 7.5-fold. In a follow-up study with highly purified human LTP, apoC-II, C-III, and E as well as apoA-I and A-II were able to stimulate LTP activity within this microemulsion assay system (Ohnishi and Yokoyama, 1993). The relationship between LTP activity and degree of saturation of lipid particle surface by apolipoproteins was maintained, while apoC-III showed less potent stimulatory effect than the other apolipoproteins.

A similar phenomenon has been observed in activation of LCAT by apoA-I or synthetic peptides with a substrate of PL unilamellar vesicles (Yokoyama *et al.*, 1980; Fukushima *et al.*, 1980). Modification of lipid particle surfaces by apolipoproteins containing multiple amphiphilic alpha-helical regions is an important mechanism for regulating LCAT activity. The same type of surface modification seems to play a role in regulating LTP activity, but enhancement of LTP activity is less specific than LCAT activation, since it could be demonstrated with a variety of apolipoproteins (Nishikawa *et al.*, 1988; Ohnishi and Yokoyama, 1993). In both cases, there is a relation between extent of binding and degree of activation, although the precise mechanism for activation remains unclear. Modulation of LTP activity through direct interaction with the enzyme LCAT (Nishida *et al.*, 1990) and apolipoproteins (Guyard-Dangremont *et al.*, 1994) has been reported. On the other hand, LTP inhibitory protein exerts its effect indirectly at the lipoprotein surface, since inhibition cannot be overcome by increasing LTP concentration (Son and Zilversmit, 1984). Apolipoproteins may also work through an indirect mechanism since there is little specificity between different apolipoproteins and the effect correlates well with degree of saturation of the microemulsion surface by the apolipoprotein. One suggestion is that apolipoproteins fill the gaps between PL molecules to decrease the energy barrier for neutral lipids to approach the lipid-water interface at the lipoprotein surface where LTP acts (Ohnishi and Yokoyama, 1993).

Reported effects of apolipoproteins on LTP activity vary greatly. ApoA-I has been reported to be stimulatory (Guyard-Dangremont *et al.*, 1994) and inhibitory (Son and Zilversmit, 1984). ApoA-II strongly stimulated LTP activity in one report (Sparks *et al.*, 1991), while others reported inhibitory effects in 2 different processes, radioisotopic lipid transfer (Guyard-Dangremont *et al.*, 1994) and HDL conversion (Rye *et al.*, 1992). However, apoA-II was not found to inhibit LTP activity *in vivo* in transgenic mice (Zhong *et al.*, 1994) or the present study. Our lab has demonstrated inhibition of LTP activity within the microemulsion assay system only when a less efficient activator, such as apoC-III, displaced a better activator, apoA-I (Ohnishi and Yokoyama, 1993). ApoC-I was slightly inhibitory in one report (Sparks *et al.*, 1991) while the N-terminal fragment of apoC-I is a strong LTP inhibitor (Kushwaha *et al.*, 1993). ApoE was found to be stimulatory, leading to increased lipoprotein binding by LTP (Kinoshita *et al.*, 1993). ApoA-IV has been reported to enhance both lipid transfer (Weinberg *et al.*, 1993) and HDL conversion (Barter *et al.*, 1988; Lagrost *et al.*, 1990) activity of LTP. It has been suggested that stimulation of LTP mediated transfer of pyrene-CE between recombinant HDL and LDL by apoA-IV operates through a so-called barostat function of apoA-IV which maintains a high lipoprotein surface pressure, limiting the surface CE concentration and facilitating lipid acceptance from LTP by lipoproteins (Weinberg *et al.*, 1994). These results also support the notion that apolipoprotein modulation of LTP activity occurs indirectly by influencing some aspect of lipoprotein structure. The effect of apoA-IV on LTP activity in our microemulsion assay system have recently been found to be comparable to apoA-I (Main *et al.*, 1996).

In this paper, we demonstrated that apoA-I and A-II, the major apolipoproteins of HDL, play a significant role in activation of LTP. Other exchangeable apolipoproteins appear to act similarly, suggesting that activation of LTP is conferred by the amphipathic alpha-helical structure of these apolipoproteins acting at the surface of lipoproteins, rather than a specific protein-protein interaction between them and LTP. Thus, modification of lipoprotein surfaces by apolipoproteins plays an important role in providing suitable characteristics at the lipid-water interface for LTP activity. Under physiological conditions, certain apolipoproteins such as apoA-IV or C-III may act to fine tune these lipoprotein surface properties. More work needs to be done to characterize their mechanism of activation, which could be through effects on surface hydrophobicity, surface pressure, or neutral lipid concentration. A good model for comparison may be LDL, which possess only apoB as its non-exchangeable apolipoprotein component, but still support LTP activity. Also, free cholesterol is an important factor influencing native lipoprotein structure and LTP activity (Morton, 1988) and its effects on LTP activity in conjunction with apolipoproteins should be evaluated in the microemulsion assay system.

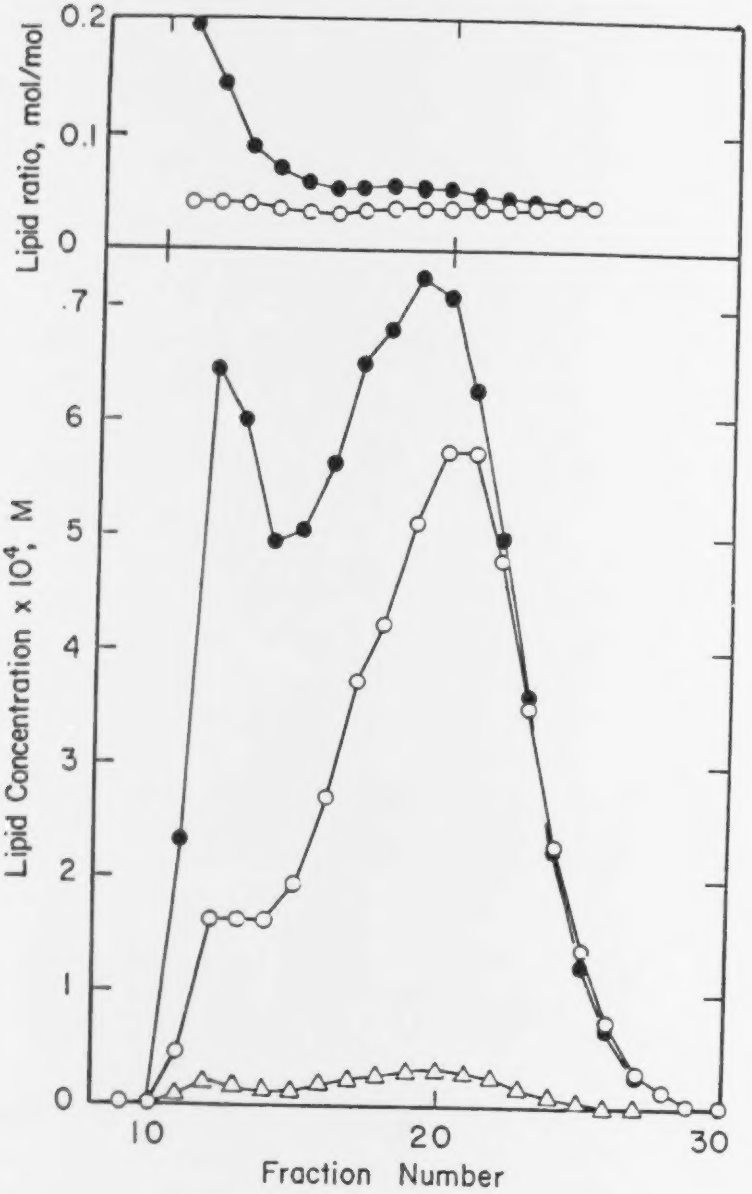


Figure 2.1. Elution profile of a lipid microemulsion preparation from a Sepharose CL-4B column. TG, PC, and pyrene-CE were mixed at a weight ratio of 5:5:1 followed by sonication, ultracentrifugation, and application to the column as described in "Experimental Procedures." In the upper panel, open circles represent the cholesterol/TG ratio, and closed circles the cholesterol/PL ratio. In the lower panel, open circles represent PC, closed circles TG, and open triangles cholesterol concentrations.

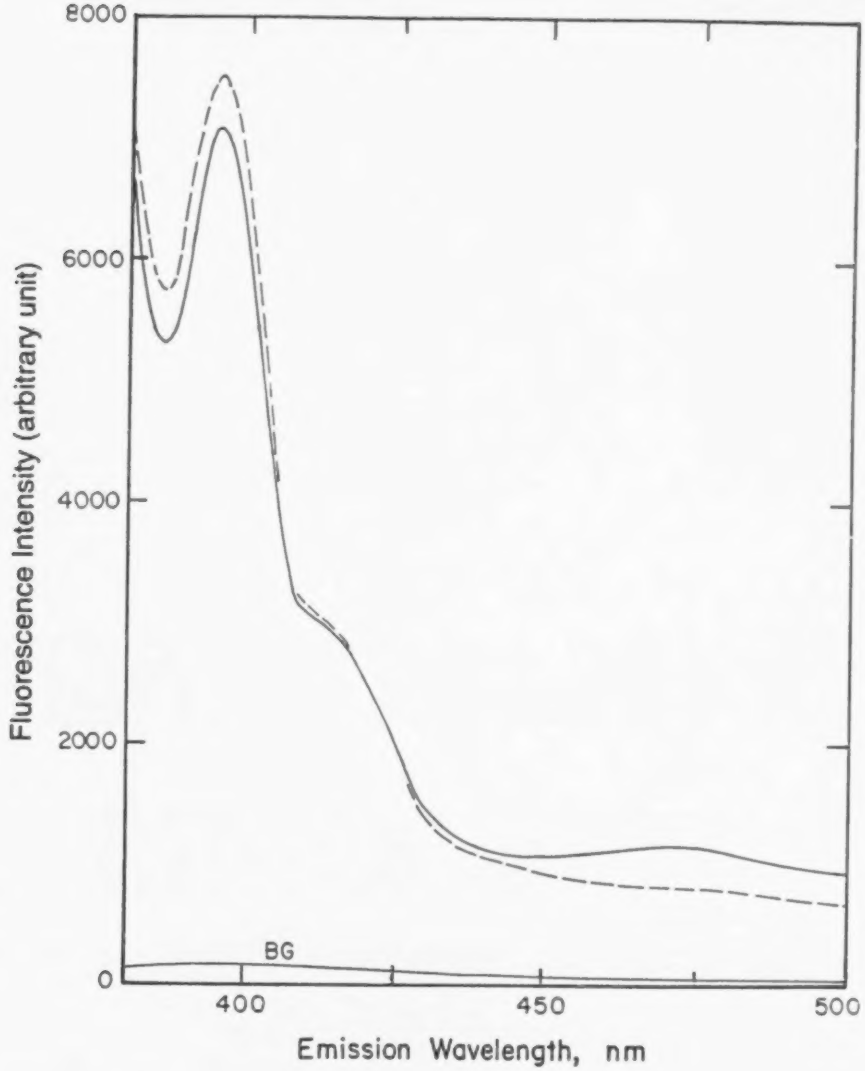


Figure 2.2. Fluorescence spectrum of lipid microemulsion particles containing pyrene-CE. The solid line was obtained with microemulsion (0.7 mM by PC) containing 2 mol% of core lipid as pyrene-CE. The broken line obtained with the same amount of microemulsion containing 0.2 mol% of core lipid as pyrene-CE. The line marked BG represents background fluorescence of microemulsion (0.9 mM by PC) without pyrene-CE.

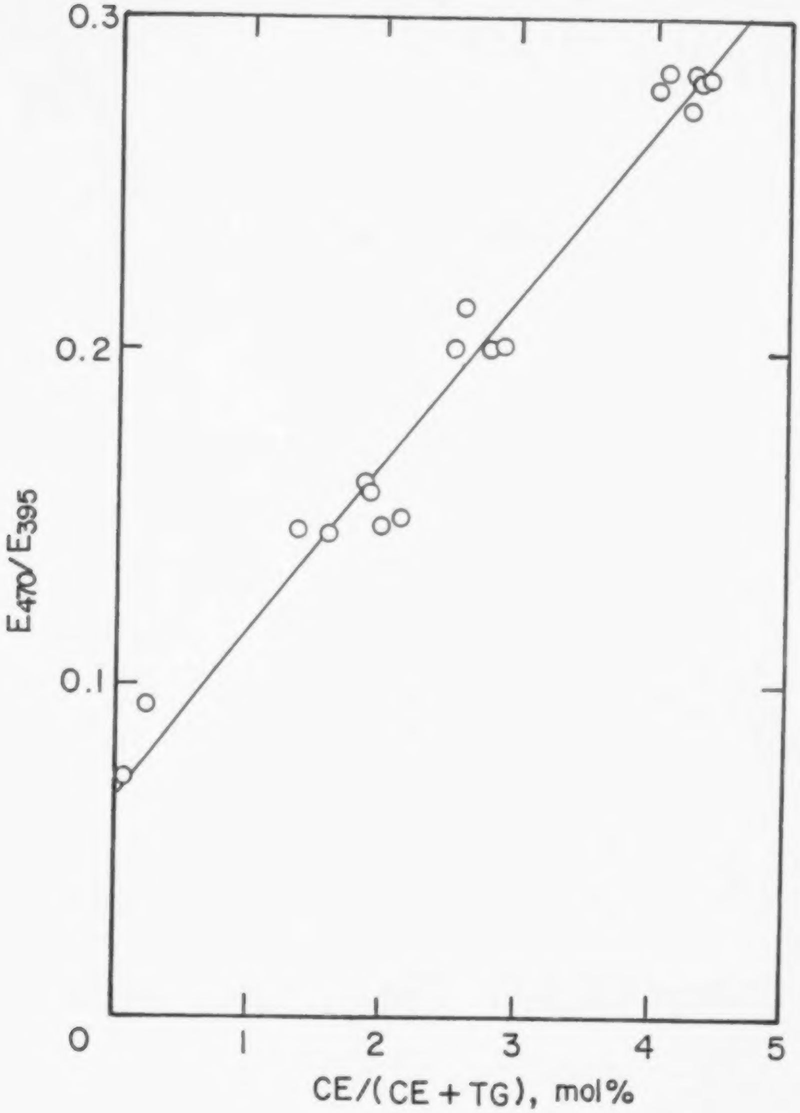


Figure 2.3. Relationship between fluorescence ratio of lipid microemulsion particles and microscopic concentration of pyrene-CE within their cores. Microemulsions with varying cholesterol content as indicated were prepared by sonication of various ratios of TG, PC, and pyrene-CE as described in "Experimental Procedures." The line was obtained by least square linear regression of the data points (slope 0.05 ± 0.02; intercept 0.066 ± 0.06).

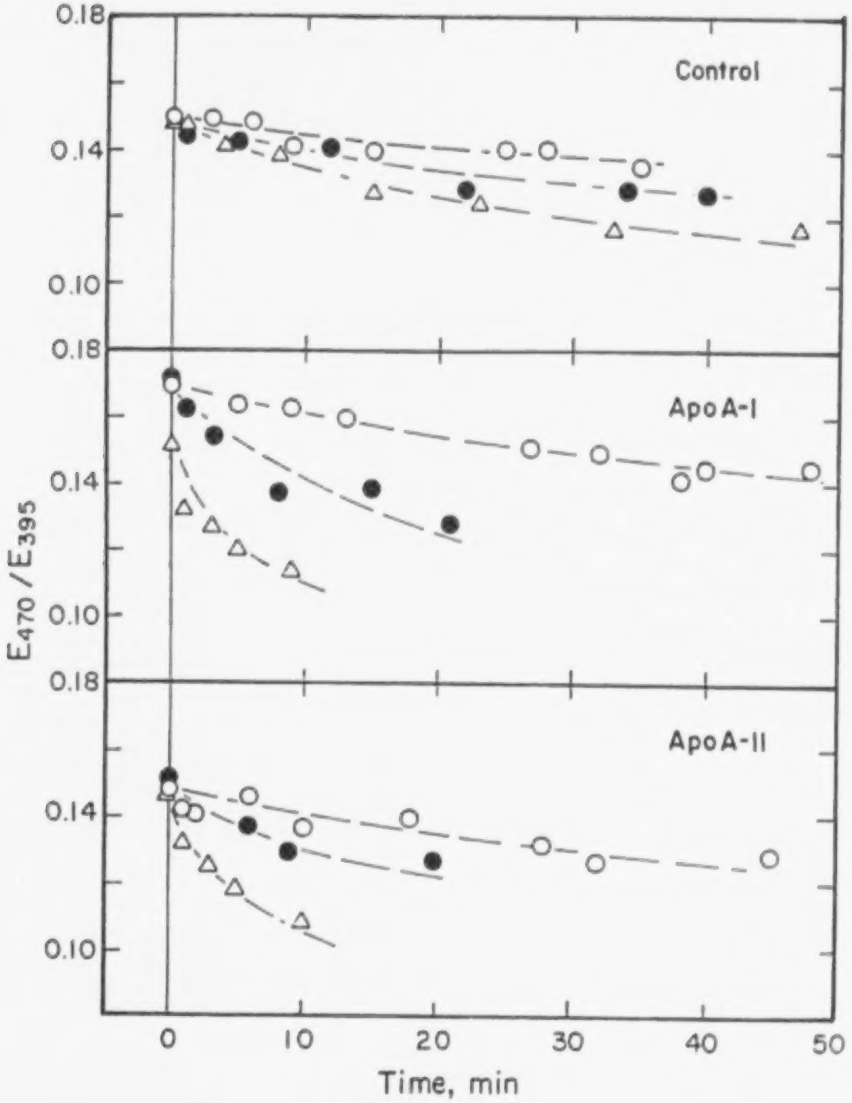


Figure 2.4. Changes in fluorescence ratio with time indicating lipid transfer. Assays were performed as described in "Experimental Procedures." The top panel is in absence of apolipoproteins, the middle panel is in presence of apoA-I (3 µM), and the bottom panel is in the presence of apoA-II (2 µM). Open circles represent spontaneous transfer in the absence of LTP. Closed circles represent transfer by of low amounts of LTP (42 µg/mL for control, 40 µg/mL for apoA-I, and 29 µg/mL for apoA-II). Open triangles represent transfer by increased amounts of LTP (100 µg/mL for control, 98 µg/mL for apoA-I, and 96 µg/mL for apoA-II).

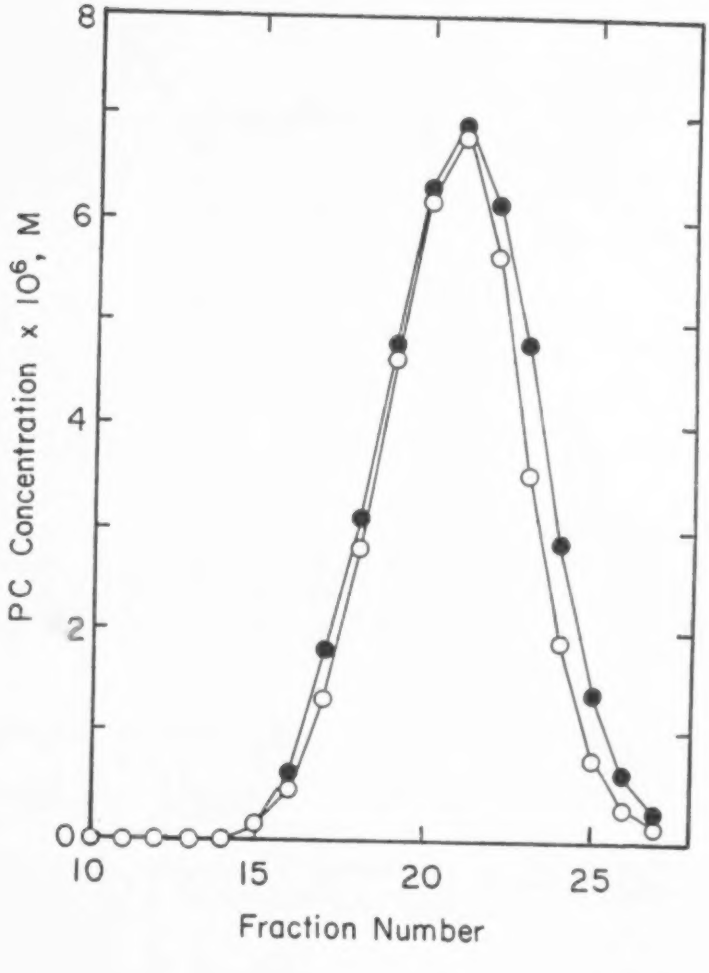


Figure 2.5. Effects of LTP and apoA-I on lipid microemulsion particle size. Shown are elution profiles of mixtures of donor and acceptor lipid microemulsion particles, 0.2 mM and 0.8 mM by PC, respectively, from a Sepharose CL-4B column. Chromatography was performed as described in Fig. 1. Open circles represent elution after a control incubation of just combined microemulsions for 30 min. Closed circles represent elution of microemulsions incubated in the presence of LTP (100 μg/mL) and apoA-I (3 μM).

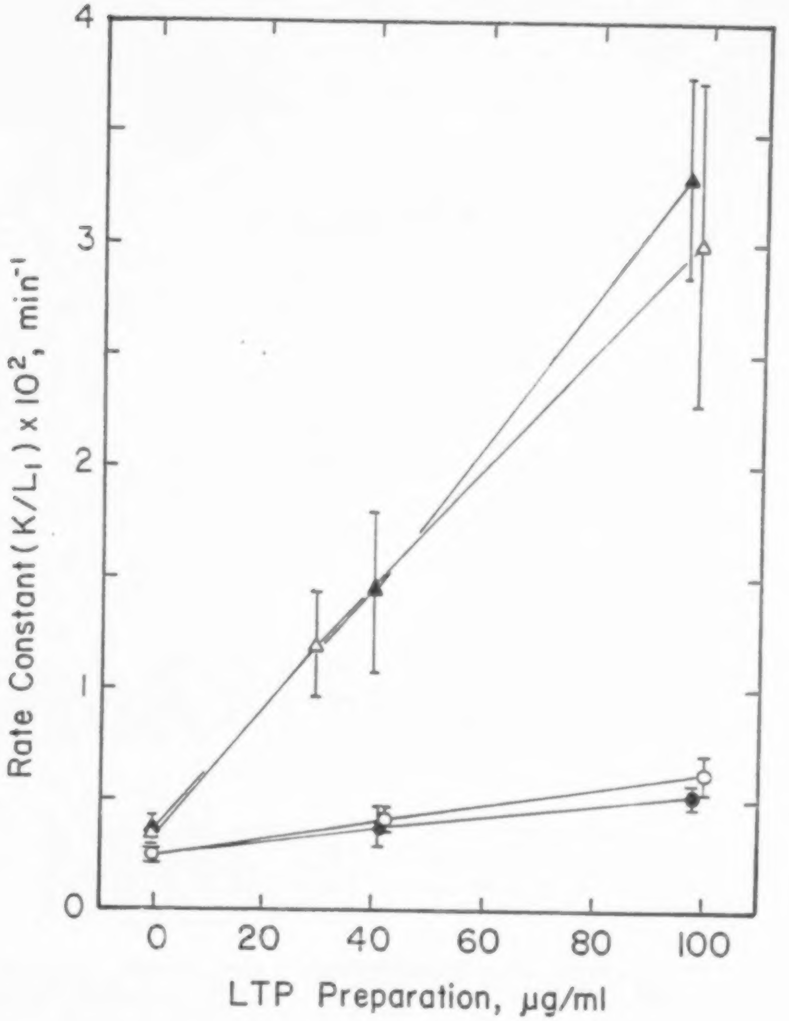


Figure 2.6. Relationships of rate constants for lipid transfer to LTP concentration. Rate constants were calculated for the data shown in Fig. 4, according to the equation given in the text. Error bars represent the standard error of the slope of the semilogarithmic plots of the data calculated by least squares linear regression. Open circles represent rate constants for control incubations without apolipoproteins; closed circles represent rate constants for controls in the presence of bovine serum albumin. Open triangles represent rate constants for lipid transfer in presence of apoA-I (3 µM); closed triangles are for rate constants in presence of apoA-II (2 µM).

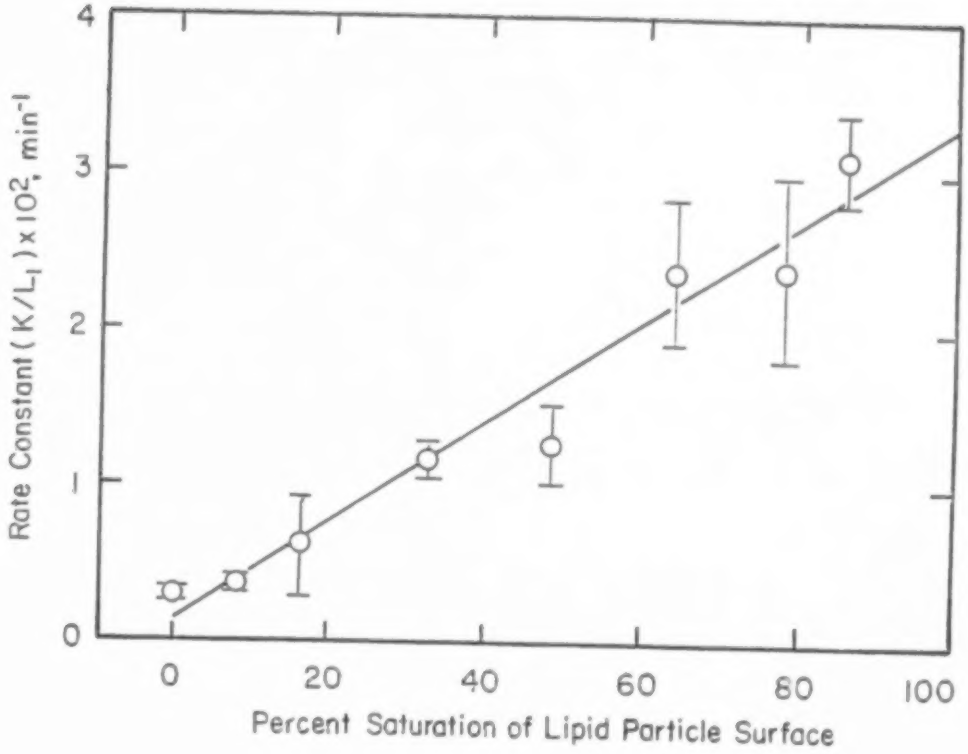


Figure 2.7. Rate constants of lipid transfer by LTP as a function of saturation of the lipid microemulsion particle surface by apoA-I. Donor microemulsions containing 4 mol% of core lipid as pyrene-CE were mixed with acceptor microemulsions at a 1:50 ratio, with a final concentration of 1.39 mM by PC. Assays were performed using 60 μg/mL LTP and varying concentrations of apoA-I to obtain rate constants. The extent of saturation of the microemulsion surface was calculated by the equation given in the text for apoA-I concentrations of 3.1×10^{-7}, 6.1×10^{-7}, 1.2×10^{-6}, 1.8×10^{-6}, 2.5×10^{-6}, 3.3×10^{-6}, and 4.0×10^{-6} M.

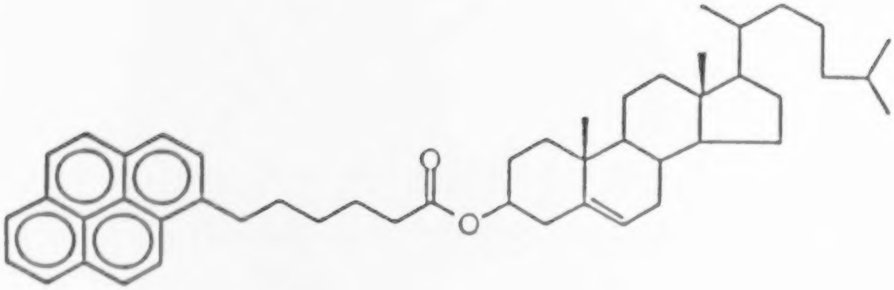


Figure 2.8. Chemical structure of pyrene-CE, or cholesterol 3-(6-(1-pyrene) hexanoate).

2.5 References

Aron, L., Jones, S., and Fielding, C.J. (1978) Human plasma lecithin-cholesterol acyltransferase. Characterization of cofactor-dependent phospholipase activity. *J. Biol. Chem.* 253, 7220-7226

Barter, P.J., and Jones, M.E. (1979) Rate of exchange of esterified cholesterol between human plasma low and high density lipoproteins. *Atherosclerosis* 34, 67-74

Barter, P.J., Rajaram, O.V., Chang, L.B.F., Rye, K.A., Gambert, P. *et al.* (1988) Isolation of a high-density-lipoprotein conversion factor from human plasma. A possible role of apolipoprotein A-IV as its activator. *Biochem. J.* 254, 179-184

Charlton, S.C., Olson, J.S., Hong, K.-Y., Pownall, H.J., Louie, D.D. *et al.* (1976) Stopped flow kinetics of pyrene transfer between human high density lipoproteins. *J. Biol. Chem.* 251, 7952-7955

Epps, D.E., Harris, J.S., Greenlee, K.A., Fisher, J.F., Marschke, C.K. *et al.* (1995) Method for measuring the activities of cholesteryl ester transfer protein (lipid transfer protein). *Chem. Phys. Lipids* 77, 51-63

Fukushima, D., Yokoyama, S., Kroon, D.J., Kezdy, F.J., and Kaiser, E.T. (1980) Chain length-function correlation of amphiphilic peptides. Synthesis and surface properties of a tetratetracontapeptide segment of apolipoprotein A-I. *J. Biol. Chem.* 255, 10651-10657

Green, S.R., and Pittman, R.C. (1991) Comparative acyl specificities for transfer and selective uptake of high density lipoprotein cholesteryl esters. *J. Lipid Res.* 32, 457-467

Guyard-Dangremont, V., Lagrost, L., and Gambert, P. (1994) Comparative effects of purified apolipoproteins A-I, A-II, and A-IV on cholesteryl ester transfer protein activity. *J. Lipid Res.* 35, 982-992

Kinnunen, P.K., Jackson, R.L., Smith, L.C., Gotto Jr., A.M., and Sparrow, J.T. (1977) Activation of lipoprotein lipase by native and synthetic fragments of human plasma apolipoprotein C-II. *Proc. Natl. Acad. Sci. USA* 74, 4848-4851

Kinoshita, M., Arai, H., Fukasawa, M., Watanabe, T., Tsukamoto, K. *et al.* (1993) Apolipoprotein E enhances lipid exchange between lipoproteins mediated by cholesteryl ester transfer protein. *J. Lipid Res* 34, 261-268

Kushwaha, R.S., Hasan, S.Q., McGill Jr., H.C., Getz, G.S., Dunham, R.G. *et al.* (1993) Characterization of cholesteryl ester transfer protein inhibitor from plasma of baboons (*Papio* sp.). *J. Lipid Res.* 34, 1285-1297

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680-685

Lagrost, L., Gambert, P., Dangremont, V., Athias, A., and Lallemant, C. (1990) Role of cholesteryl ester transfer protein (CETP) in the HDL conversion process as evidenced by using anti-CETP monoclonal antibodies. *J. Lipid Res.* 31, 1569-1575

Main, L. A., Ohnishi, T., and Yokoyama, S. (1996) Activation of human plasma cholesteryl ester transfer protein by human apolipoprotein A-IV. *Biochim. Biophys. Acta* 1300, 17-24

Massey, J.B., Gotto Jr., A.M., and Pownall, H.J. (1982) Kinetics and mechanism of the spontaneous transfer of fluorescent phosphatidylcholines between apolipoprotein-phospholipid recombinants. *Biochemistry* 21, 3630-3636

Morton, R.E. (1986) Specificity of lipid transfer protein for molecular species of cholesteryl ester. *J. Lipid Res.* 27, 523-529

Morton, R.E. (1988) Free cholesterol is a potent regulator of lipid transfer protein function. *J. Biol. Chem.* 263, 12235-12241

Morton, R.E., and Zilversmit, D.B. (1982) Purification and characterization of lipid transfer protein(s) from human lipoprotein-deficient plasma. *J. Lipid Res.* 23, 1058-1067

Morton, R.E., and Zilversmit, D.B. (1983) Inter-relationship of lipids transferred by the lipid-transfer protein isolated from human lipoprotein-deficient plasma. *J. Biol. Chem.* 258, 11751-11757

Nishida, H.I., Kato, H., and Nishida, T. (1990) Affinity of lipid transfer protein for lipid and lipoprotein particles as influenced by lecithin-cholesterol acyltransferase. *J. Biol. Chem.* 265, 4876-4883

Nishide, T., Tollefson, J.H., and Albers, J.J. (1989) Inhibition of lipid transfer by a unique high density lipoprotein subclass containing an inhibitor protein. *J. Lipid Res.* 30, 149-158

Nishikawa, O., Yokoyama, S., Okabe, H., and Yamamoto, A. (1988) Enhancement of non-polar lipid transfer reaction through stabilization of substrate lipid particles with apolipoproteins. *J. Biochem.* 103, 188-194

Ohnishi, T. and Yokoyama, S. (1993) Activation of human plasma lipid transfer protein by apolipoprotein. *Biochemistry* 32, 5029-5035

Ohnishi, T., Yokoyama, S., and Yamamoto, A. (1990) Rapid purification of human lipid transfer proteins. *Biochemistry* 31, 397-406

Ohnishi, T., Tan, C., and Yokoyama, S. (1994) Selective transfer of cholesteryl ester over triglyceride by human plasma lipid transfer protein between apolipoprotein-activated lipid microemulsions. *Biochemistry* 33, 4533-4542

Okabe, H., Yokoyama, S., and Yamamoto, A. (1988) Modulation of cholesterol microenvironment with apolipoproteins induced by the presence of cholesteryl ester in lipid microemulsion. *J. Biochem.* 104, 141-148

Pattnaik, N.M., Montes, A., Hughes, L.B., and Zilversmit, D.B. (1978) Cholesteryl ester exchange protein in human plasma. Isolation and characterization. *Biochim. Biophys. Acta* 530, 428-438

Pownall, H.J. and Smith, L.C. (1973) Viscosity of the hydrocarbon region of micelles. Measurement of excimer fluorescence. *J. Am. Chem. Soc.* 95, 3136-3140

Pownall, H.J., and Smith, L.C. (1989) Pyrene-labeled lipids: dynamics *in vitro* and in living cells. *Chem. Phys. Lipids* 50, 191-211

Roseman M.A. and Thompson, T.E. (1980) Mechanism of the spontaneous transfer of phospholipids between bilayers. *Biochemistry* 19, 439-444

Rye, K.-A., Garrety, K.H., and Barter, P.J. (1992) Changes in the size of reconstituted high density lipoproteins during incubation with cholesteryl ester transfer protein: the role of apolipoproteins. *J. Lipid Res.* 33, 215-224

Son, Y.-S.C., and Zilversmit, D.B. (1984) Purification and characterization of human plasma proteins that inhibit lipid transfer activities. *Biochim. Biophys. Acta* 795, 473-480

Sparks, D., Frohlich, J.J., and Pritchard, P.H. (1991) Lipid transfer proteins, hypertriglyceridemia, and reduced high-density lipoprotein cholesterol. *Am. Heart J.* 122, 601-607

Swenson, T.L., Brocia, R.W., and Tall, A.R. (1988) Plasma cholesteryl ester transfer protein has binding sites for neutral lipids and phospholipids. *J. Biol. Chem.* 263, 5150-5157

Swenson, T.L., Hesler, C.B., Brown, M.L., Quinet, E., Trotta, P.P. *et al.* (1989) Mechanism of cholesteryl ester transfer protein inhibition by a neutralizing monoclonal antibody and mapping of the monoclonal anytibody epitope. *J. Biol. Chem.* 264, 14318-14326

Tajima, S., Yokoyama, S., and Yamamoto, A. (1983) Effect of lipid particle size on association of apolipoproteins with lipid. *J. Biol. Chem.* 258, 10073-10082

Thuren, T., Wilcox, R.W., Sisson, P., and Waite, M. (1991) Hepatic lipase hydrolysis of lipid. Regulation by apolipoproteins. *J. Biol. Chem.* 266, 4853-4861

Weinberg, R.B., Hopkins, R.A., Kussie, P., and Tall, A.R. (1993) Apolipoprotein A-IV accelerates the transfer of cholesterol ester by recombinant human cholesteryl ester transfer protein. *Circulation* 88, I461

Weinberg, R.B., Cook, V.R., Jones, J.J., Kussie, P., and Tall, A.R. (1994) Interfacial properties of recombinant human cholesterol ester transfer protein. *J. Biol. Chem.* 269, 29588-29591

Weisgraber, K.H. (1994) Apolipoprotein E: structure-function relationships. *Adv. Protein Chem.* 45, 249-302

Windler, E., and Havel, R.J. (1985) Inhibitory effects of C apolipoproteins from rats and humans on the uptake of triglyceride-rich lipoproteins and their remnants by the perfused rat liver. *J. Lipid Res.* 26, 556-565

Yang, C.-Y., Chen, S.-H., Bradley, W.A., Sparrow, J.T., Tanimura, M. *et al.* (1986) Sequence, structure, receptor-binding domains and internal repeats of human apolipoprotein B. *Nature* 323, 738-742

Yao, Z., and McLeod, R.S. (1994) Synthesis and secretion of hepatic apolipoprotein B-containing lipoproteins. *Biochim. Biophys. Acta* 12112, 152-166

Yokoyama, S. (1990) Self-associated tetramer of human apolipoprotein E does not lead to its accumulation on a lipid particle. *Biochim. Biophys. Acta* 1047, 99-101

Yokoyama, S., Fukushima, D., Kupferberg, J.P., Kezdy, F.J., and Kaiser, E.T. (1980) The mechanism of activation of lecithin:cholesterol acyltransferase by apolipoprotein A-I and an amphiphilic peptide. *J. Biol. Chem.* 255, 7333-7339

Yokoyama, S., Tajima, S., and Yamamoto, A. (1982) The process of dissolving apolipoprotein A-I in an aqueous buffer. *J. Biochem.* 91, 1267-1272

Zhong, S., Goldberg, I.J., Bruce, C., Rubin, E., Breslow, J. (1994) Human apoA-II inhibits the hydrolysis of HDL triglyceride and the decrease of HDL size induced by hypertriglyceridemia and cholesteryl ester transfer protein in transgenic mice. *J. Clin. Invest.* 94, 2457-2467

CHAPTER 3

Purification of Rabbit LTP and Characterization of its Interaction with Lipid Microemulsion Particles[1]

3.1 Introduction

Plasma LTP activity is species-specific, with humans and rabbits possessing significant levels (Ha and Barter, 1982). The protein encoded by the cDNA of rabbit LTP is longer than its human counterpart by 20 amino acids, with 19 residues comprising a single insertion within the C-terminal region of rabbit LTP (Nagashima *et al.*, 1988). The overall primary sequence of LTP is highly conserved between rabbit and human LTP, including the C-terminal 26 residues, which form the epitope of a human LTP inhibitory mAb, TP2, as well as being essential for neutral lipid binding and transfer by human LTP (Swenson *et al.*, 1989). Some functional differences between rabbit and human LTP may exist. While human LTP is mostly associated with lipoproteins within plasma (Marcel *et al.*, 1990; Moulin *et al.*, 1994), most of rabbit LTP is free (Tall *et al.*, 1987). Rabbit LTP does not exhibit specificity towards neutral lipids, while human LTP prefers to transfer CE over TG (Morton and Zilversmit, 1983). Furthermore, the levels of CE transfer activity are higher in rabbit than human plasma (Ha and Barter, 1988).

The mechanism of neutral lipid transfer between lipoproteins across an aqueous barrier by LTP is unclear, with kinetic evidence supporting both carrier (Barter and Jones, 1980) or ternary complex formation (Ihm *et al.*, 1982) schemes. Various results have provided additional support for either of these hypotheses. Direct demonstration of neutral lipid binding and carrying capacity of LTP (Swenson *et al.*, 1988) and studies employing PL monolayers (Weinberg *et al.*, 1994) indicate that LTP can operate through a carrier mechanism. Meanwhile, increased LTP activity in association with increased lipoprotein binding has been observed by increasing LTP concentration (Morton, 1985), or addition of free fatty acid (Sammett and Tall, 1985), apoE

[1] A version of this chapter has been published: Ko, K.W.S., Oikawa, K., Ohnishi, T., Kay, C.M., and Yokoyama, S. (1993) *Biochemistry* 32, 6729-6736.

(Kinoshita *et al.*, 1993), or LCAT (Nishida *et al.*, 1990) to an assay system, possibly supporting a ternary complex mechanism.

We have recently devised a system for studying the activation of LTP by apolipoproteins, employing lipid microemulsion particles carrying pyrene-CE as a transfer probe (Milner *et al.*, 1991 and Chapter 2). In the present study, we set out to isolate mammalian plasma LTP for carrying out investigations on the mechanism of LTP mediated neutral lipid transfer. Rabbit plasma provides an abundant source of LTP activity for isolation purposes. A rapid and efficient purification procedure was developed, starting with frozen, commercially available rabbit plasma. Molecular characterization of the purified LTP enabled us to verify its identity and contrast several features with those of human LTP. The behavior of rabbit LTP in the presence of lipid microemulsion particles was analyzed by a binding assay as well as CD spectroscopy, providing further details about the mechanism of LTP mediated neutral lipid transfer within our microemulsion assay system.

3.2 Materials and Methods

3.2.1 Materials

Aprotinin (Trasylol) and gentamycin (Garamycin) were purchased from Miles and Schering, respectively. [1α, 2α (n)-^{3}H]Cholesteryl oleate was purchased from Amersham. Cholesteryl (pyren-1-yl)-hexanoate and triolein (>99% pure) were purchased from Sigma. Egg PC was purchased from Avanti Polar Lipids. Dextran sulfate-cellulose (Yokoyama *et al.*, 1984) was kindly provided by Kanegafuchi Chemical Industries, Osaka. Enzymatic total and free cholesterol and TG assay kits were purchased from Wako Pure Chemicals, Richmond, VA. Frozen rabbit plasma was purchased from Pel-freez, Rogers, AR. Toyopearl Butyl-650M and carboxymethyl (CM)-650M gels were purchased from Supelco Canada. Ampholytes were from purchased Pharmacia. TP2 mAb against human LTP was a generous gift from Drs. Yves Marcel and Ross Milne, Ottawa Heart Institute (Hesler *et al.*, 1988). ApoA-I isolated from human HDL as previously described (Nishikawa *et al.*, 1988) and human LTP (Ohnishi *et al.*, 1990) were kindly provided by Dr. Taira Ohnishi of our laboratory. All other chemicals and materials were reagent grade available from Sigma, Bio-Rad or other suppliers as stated.

3.2.2 Lipoprotein Preparation

All centrifugations and further procedures were performed at 4° C, except where stated otherwise. For preparation of either HDL or [^{3}H]cholesteryl oleate labeled LDL, fresh human plasma was obtained by collecting blood from overnight fasted volunteers into 0.1% (w/v) EDTA, followed by sequential centrifugations at 2,500 rpm (500 x *g*) and 6,000 rpm (2,800 x *g*) for 15 min each (Beckman JA-20). Sodium azide (0.02%, w/v), aprotinin (20 U/mL), DTNB (2 mM), and gentamycin (0.1 mg/mL) were then added.

HDL was isolated from fresh human plasma by preparative ultracentrifugal flotation with density adjustment by addition of solid NaBr (Hatch & Lees, 1968). 200 mL of plasma was adjusted to d = 1.063 g/mL by addition of 14.32 g NaBr followed by ultracentrifugation in Quick-Seal polyallomer tubes at 50,000 rpm (184,000 x *g*) for 40 h (Beckman 70 Ti). The top layer of VLDL and LDL (48 mL) was removed with a syringe needle and the infranatant and bottom gelatinous material were resuspended by stirring for 2 h. The mixture (147 mL) was spun briefly at 6,000 rpm (2,800 x *g*) for 5 min (Beckman JA-20) and then adjusted to d = 1.21 g/mL by addition of 28.5 g NaBr, followed by ultracentrifugation in Quick-Seal tubes at 60,000 rpm (265,000 x *g*) for 40 h (Beckman 70 Ti). The top layer of HDL (26 mL) was removed with a syringe needle and dialyzed against 2 L of 10 mM sodium phosphate, pH 7.4, 150 mM NaCl, 0.02% (w/v) sodium azide (LTP buffer) plus 1 mM EDTA for 48 h, changing the buffer once halfway through.

[^{3}H]Cholesteryl oleate labeled LDL preparation was based on the method of Nishikawa *et al.* (1986). One hundred fifty mL human plasma was spun at 50,000 rpm (184,000 x *g*) for 16 h (Beckman 70 Ti) and the floated VLDL (30 mL) was removed with a syringe needle. The infranatant and gelatinous pellet were resuspended by stirring for 3 h. During this time, radiolabeled vesicles were prepared by drying down 4.5 mg egg PC and 500 μL [^{3}H]cholesteryl oleate in a test tube with N_2, followed by sonication in 2 mL of LTP buffer, with the sample placed on ice and under a stream of N_2, for 15 min (Heat Systems XL 2020 using a Microtip) until clear. The radioactive vesicles were combined with the VLDL-depleted human plasma and the mixture was incubated in a 37° C water bath for 24 h, then centrifuged at 2,500 rpm (2,000 x *g*) for 15 min (Beckman JA-20). The supernatant was applied (1 mL/min) onto a dextran sulfate-cellulose column (made from a 50 mL syringe) equilibrated with LTP buffer. After extensive washing with LTP buffer, the retained LDL was eluted with LTP buffer plus 0.35 M NaCl . The LDL containing fractions were pooled visually (yellow color, 50 mL) and adjusted to *d* = 1.07 g/mL by addition of 4.09 g NaCl, followed by ultracentrifugation in polycarbonate tubes at 100,000 rpm (412,000 x *g*) for 2.5 h (Beckman TLA-100.3). The floated radiolabeled LDL (12 mL) was removed with a Pipetman P200 (Gilson) and dialyzed as described above for HDL.

Total and free cholesterol content of the prepared lipoproteins were measured by enzymatic assay kits according to the manufacturers instructions. A typical preparation of radiolabeled LDL was 17.2 mM in CE (22.8 mM total cholesterol) and labeled to a specific radioactivity of 1,800 dpm/nmol in CE, while HDL was 6.4 mM in CE (7.9 mM total cholesterol). Prepared lipoproteins were stored in small aliquots at -70° C until use.

3.2.3 Assays of LTP Activity

LTP activity as transfer of radiolabeled CE from LDL to HDL was measured as previously described (Ohnishi *et al.*, 1990), except that the reaction volumes were halved and incubations were carried out for 3 h. Thus, the assay mixture contained HDL and radiolabeled LDL (40 and 47.5 μg cholesterol moiety, respectively), the sample being assayed, 6% (w/v) bovine serum albumin (fatty acid-free, Sigma) and LTP buffer, in a final volume of 237.5 μL. The assays were carried out in 1.5 mL plastic vials (Eppendorf) which were placed in a heated water bath at 37° C for 3 h. The assay was stopped by addition of 12.5 μL of 5% (w/v) heparin, 1 M $MnCl_2$, followed by vortexing and incubation on ice for 30 min before centifugation at 13,000 rpm for 15 min at 4° C (Beckman Microfuge 11). Aliquots (100 μL) of reaction supernatant were placed into counting vials (Minivial) along with 5 mL ACS (Amersham) and counted in a Beckman LS6000TA scintillation counter. Activity values were determined only using data falling within the linear region of a curve of counts versus sample protein. Typically then, sample data resulted in less than 18% of donor label being transferred, while blanks exhibited about 0.04% of donor label transferred. Under the conditions used to precipitate donor and stop the assay, a considerable amount of HDL is also found to be precipitated; however, such conditions were necessary to minimize background counts from radiolabeled donor LDL. The amount of

HDL recovered in the supernatant was found to be reproducible (30%), and transfer activity values in Table 3.1 have been corrected for HDL recovery.

Alternatively, CE transfer activity was measured between lipid microemulsion particles of TG and egg PC (1:1.4 by weight) using pyrene-labeled CE as a transfer probe (Ohnishi and Yokoyama, 1993). Donor microemulsion containing pyrene-CE (5.3 mol% of core lipid) and acceptor microemulsion without pyrene-lipid, 0.8 and 7.2 µg in PC respectively, were combined with 4 µg human apoA-I and then the reaction was initiated by adding 1 µg LTP in a final incubation volume of 0.4 mL (LTP concentration was 47 nM). The reaction was monitored as the decrease in ratio of excimer to monomer fluorescence emission peak intensities at 37° C in a Hitachi F-2000 fluorescence spectrophotometer equipped with a thermostatic cell holder and a micromagnetic stirring unit. The rate constant of pyrene-lipid transfer, k/L_1 (L_1 being donor lipid poolsize), was calculated according to the previously described equation (Milner *et al.*, 1991 and Chapter 2).

3.2.4 Purification of Rabbit LTP

Frozen rabbit plasma (1.5 L) was thawed in a water bath at 37° C and then adjusted to 1 mM phenylmethylsulfonyl flouride (PMSF), 2 mM leupeptin, 1 mM benzamidine, and 1 µM pepstatin A. All following procedures were performed at 4° C. This was followed by the addition of $MnCl_2$ to reach 0.2 M and 96 mL of 10% (w/v) dextran sulfate (final concentration 0.6%) followed by stirring for 10 min. The supernatant was isolated by centrifugation at 10,000 rpm (9,600 x *g*) for 20 min (Beckman JA-14). Addition of $BaCl_2$ to 1%, followed by stirring for 10 min and another spin under the same conditions to further remove lipoprotein complexes gave lipoprotein-depleted plasma. NaCl was added to increase the concentration by 2 M before application to the first column.

The lipoprotein-depleted plasma, plus 2 M NaCl was loaded onto a Butyl-Toyopearl column (5 cm i.d. x 14 cm) equilibrated with 2 M NaCl at 5 mL/min (Fig. 3.1, panel A). The column was washed with an equal volume of 50 mM NaCl, 2 mM EDTA, 1 mM PMSF (position 1). Lipid transfer activity was eluted beginning with an 800 mL gradient of 50 mM NaCl, 2 mM EDTA, 1 mM PMSF to distilled water (position 2) followed by straight water (position 3). Fifteen mL fractions were collected and 50 µL aliquots across the protein peak were assayed for lipid transfer activity. The tubes containing activity were pooled and adjusted to 20 mM sodium citrate by the addition of one-quarter volume of 100 mM sodium citrate (pH 5.5) before application to the next column. In between purifications, the column packing was regenerated by washing with 2 M NaOH and extensive rinsing with water.

A CM-Toyopearl column (2.8 cm i.d. x 20 cm) was equilibrated with first 100 mM sodium citrate (pH 5.5) and then 20 mM sodium citrate (pH 5.5) until the effluent was pH 5.5. The pooled active fraction from the previous step, adjusted to 20 mM sodium citrate (pH 5.5), was loaded at 4 mL/min (Fig. 3.1, panel B). Then the column was washed with 20 mM sodium citrate (pH 5.5) plus the following protease inhibitors: 1 mM EDTA, 1 mM PMSF, 1 mM benzamidine, 1 μM pepstatin (position 1) until the UV absorbance returned to near baseline. Lipid transfer activity was eluted beginning with a sharp 50 mL gradient of 0-1 M NaCl in 20 mM sodium citrate plus protease inhibitors (position 2) continued with the 1 M NaCl buffer (position 3). Ten mL fractions were collected and 20 μL aliquots (excessive amounts of high salt-containing samples interfere with LDL precipitation in the LTP assay) across the protein peak were assayed for lipid transfer activity before pooling the active fractions. In between purifications, the column packing was washed with 0.1 M NaOH, water, 0.5 M HCl and extensive rinsing with water.

A small Butyl-Toyopearl column (2.3 cm i.d. x 7 cm) was equilibrated with 1 M NaCl, 1 mM EDTA, 1 mM PMSF (equilibration buffer). The pooled active fraction from the previous step (80 mL) was loaded directly onto the column at 1.5 mL/min (Fig. 3.1, panel C) followed by an equal volume of equilibration buffer (position 1). The column was washed with 150 mM NaCl, 1 mM EDTA, 1 mM PMSF (position 2) until the UV absorbance returned to near baseline and further with three column volumes of 1 mM EDTA, 1 mM PMSF (position 3) before lipid transfer activity was eluted with distilled water (position 4). Three mL fractions were collected and 10 μL aliquots across the protein peak were assayed for lipid transfer activity. In between purifications, the column packing was washed as described above.

3.2.5 Electrophoresis and Immunoblotting

Polyacrylamide gel electrophoresis (PAGE) in the presence of 0.1% (w/v) SDS was carried out according to the method of Laemmli (1970) using a 12% acrylamide separating gel in a vertical slab mini-gel apparatus (Atto, Japan). Before application, samples were heated at 90° C in the presence of 1%

(w/v) SDS and 2.5% (v/v) mercaptoethanol and some samples were desalted or concentrated by deoxycholate-trichloroacetic acid precipitation (Peterson, 1983). Protein bands were visualized by staining with 0.2% (w/v) Coomassie Blue R250 in 40% methanol/10% acetic acid (both v/v) for 1 h and destaining with 10% (v/v) acetic acid.

Native isoelectric focusing was performed by mini-gel using a 6% total/0.96% cross-linker acrylamide (both w/v) gel containing 0.4% (v/v) Ampholine 3.5-10, 2% (v/v) Ampholine 4-6 and 0.1% (w/v) tetramethylethylenediamine. A 20 mL volume of gel solution was polymerized by degassing and addition of 0.6% (w/v) ammonium persulfate. Samples were taken up in equal volumes of sample buffer containing 15% (w/v) glycerol, 0.8% (v/v) Ampholine 3.5-10, 4% (v/v) Ampholine 4-6 and 0.04% (w/v) bromophenol blue. The catholyte (upper) was 20 mM NaOH and the anolyte (lower) was 10 mM phosphoric acid; focusing was performed at 200 V constant voltage for 1.5 h and then 400 V for 1.5 h. The pH gradient was measured by cutting a gel lane into slices down its length and soaking these pieces in 1 mL of 10 mM KCl for 1 h before measurement by pH meter.

Protein bands were detected after isoelectric focusing, and in some cases after SDS-PAGE, by immunoblotting. This was performed by soaking the gel in 25 mM Tris-base, 192 mM glycine, 20% (v/v) methanol followed by electrophoretic transfer of proteins to nitrocellulose membrane (Bio-Rad) for 2 h at 200 mA. The membrane was then blocked for 2 h with 20 mM Tris (pH 7.4), 0.5 M NaCl (Tris buffered saline, TBS) plus 5% (w/v) dry milk and soaked twice with TBS plus 0.05% (w/v) Tween (Wash buffer) for 5 min before incubation for 2 h with TP2 antibody diluted 1:500 in Wash buffer plus 2% (w/v) milk powder (Antibody buffer). The membrane was then washed twice with Wash buffer before incubation for 1 h with horseradish peroxidase-linked sheep anti-mouse immunoglobulin (Amersham) diluted 1:10,000 in Antibody buffer. The membrane was then washed twice with Antibody buffer, twice with Wash buffer and three times with TBS. For fluorography, the membrane was then treated with Gene Detection Reagents 1 and 2 (Amersham) (1:1 mixture) for 1 min before covering in Parafilm and exposing to XAR 5 film (Kodak) which was developed by automated processor (Fuji).

3.2.6 Gel Permeation Chromatography

Gel permeation chromatography was performed using a Superose 12 HR10/30 column connected to a standard FPLC (Pharmacia) apparatus at 4° C. LTP buffer was the mobile phase at a flow rate of 0.75 mL/min. Monitoring the elution by absorbance at 280 nm, 200 µL samples of globular protein molecular weight standards catalase (220 kDa), aldolase (158 kDa), transferrin (81 kDa), albumin (66 kDa), ovalbumin (43 kDa) and soybean trypsin inhibitor (22.1 kDa) were markers to calibrate K_{av} versus log molecular mass. The same volume of pure rabbit LTP was then analyzed with elution monitored by LTP assay and immunoblotting after SDS-PAGE of the column fractions.

3.2.7 Protein Sequencing and Amino Acid Analysis

Purified rabbit LTP was sequenced and analyzed for a fee by the Alberta Peptide Institute, University of Alberta. N-terminal sequencing was done on an Applied Biosystems 473A sequencer. Approximately 100 pmol of sample in water was applied directly onto a Biobrene-treated and trifluoroacetate-washed glass fiber filter which was then subjected to automated Edman degradation. PTH derivatives of the amino acids were analyzed by an on-line reverse phase high performance liquid chromatography system.

Amino acid analysis was carried out on aliquots of sample which were dried down in blast furnace treated glass tubes and then resuspended in 6 M HCl. The tubes were evacuated and sealed and hydrolysis was carried out for 24 h at 110° C before analysis against reference amino acids separated by cation exchange chromatography and detected by post column ninhydrin reaction using a Beckman 6300 analyzer.

3.2.8 Lipid Microemulsion Particle Binding Analysis

Lipid microemulsion particles were prepared as previously described (Milner *et al.*, 1991 and Chapter 2). The final material had a lipid concentration of 1.9 mg/mL in TG and a TG/PC weight ratio of 1:1.36. Binding of rabbit LTP was assessed by incubating pure LTP in LTP buffer, 0.5% albumin (Sigma) with or without microemulsion (65 µg by TG) for 15 min at 37° C. Then the sample in 200 µL was ultracentrifuged at 100,000 rpm (412,000 x *g*) for 15 min at 25° C (Beckman TLA-100). After completion of the spin, the bottom 100 µL of solution was removed with a microsyringe immersed from above to the tube bottom. LTP content of the separated halves was determined by measurement of LTP activity and interpolation from the linear region of a standard curve of LTP activity versus pure protein, and lipid content was determined by enzymatic TG assay kit according to the manufacturer's instructions.

3.2.9 CD Spectroscopy

Measurements were carried out on a Jasco J-720 spectropolarimeter at room temperature. The instrument was calibrated with d(+)-10-camphorsulfonic acid at 290.5 nm and 192 nm and d(-)-pantoyllactone at 219 nm. LTP (72 µg/mL) in 10 mM sodium phosphate (pH 7.4), 150 mM NaCl was placed in a temperature controlled 0.05 cm cell at 25° C. Ten scans were performed and signal due to solvent was subtracted. Spectra were also measured in the presence of lipid microemulsion particles same as those used for binding studies (above). Molar ellipticities were calculated according to the equation $[q] = q_{obs}/(10\ x\ l\ x\ C)$ where $[q]$ is molar ellipticity in degree-cm squared per dmol, q_{obs} is observed ellipticity in degrees, l is pathlength in cm, and C is concentration in mg/mL/109.65 (109.65 being the mean residue weight of LTP). The ellipticity-versus-wavelength data were analyzed by a computer program (CONTIN version 1.0) according to a method (Provencher & Glöckner, 1981) for prediction of secondary structure from CD spectra based on 16 proteins of known structure from x-ray crystallography. No correction for carbohydrate content was included in analysis of the CD spectra.

3.2.10 Other Methods

Protein concentrations were routinely determined by the method of Lowry (Lowry *et al.*, 1951) with bovine serum albumin as a standard and by amino acid analysis for Ala and Leu content of LTP for the CD studies. The results from either method were similar.

3.3 Results

3.3.1 Purification of Rabbit LTP

Using radioisotopic CE transfer to monitor elution of activity, rabbit LTP was purified approximately 4,000-fold from rabbit plasma (Table 3.1). The elution profile of lipid transfer activity from the Butyl-Toyopearl column coincided with the single, large protein peak retained by the column and eluted with water (Fig. 3.1, panel A). Elution of activity from the next step, CM-Toyopearl, also coincided with a major absorbance peak of retained protein eluted by high salt buffer (Fig. 3.1, panel B). The later eluting peak was absent in runs without PMSF or benzamidine, and thus may not be due to protein. Resolution of the peak components was not improved by shallowing the salt gradient, as the activity peak also broadened. Following direct application of the active fraction from the previous step onto a small Butyl-Toyopearl column, LTP activity was eluted as a single peak in water (Fig. 3.1, panel C). The lower salt concentration during application of the sample in the second Butyl-Toyopearl column may have contributed to the significant purification achieved by this step. The CM-Toyopearl step in between could not be omitted, otherwise many impurities remained in the following step. The recoveries from each Butyl-Toyopearl step was only about 20%, although recovery of LTP following precipitation of lipoproteins from plasma could not be determined due to interference of the assay by this fraction.

SDS-PAGE of the active fractions obtained at each step of purification showed that the fraction after the final step consisted of a single, broad band of M_r = 74,000 (Fig. 3.2). In contrast to purification of human LTP, where LTP appeared to be the major band after CM-Toyopearl (Ohnishi *et al.*, 1990), rabbit LTP was only resolved from a major lower M_r contaminant in the final step. Inclusion of protease inhibitors in the column buffers during the isolation procedure was essential for intactness of this band. Verification that this band was LTP was obtained by its crossreactivity during immunoblotting with TP2, a mAb against human LTP (Hesler *et al.*, 1988) which recognized a single band of M_r = 74,000 in both human (not shown) and rabbit plasma, as well as the major band isolated by our purification procedure (Fig. 3.3).

3.3.2 Molecular Characterization of Rabbit LTP

N-terminal sequencing of our protein without chemical modification of Cys residues identified 13 of the first 15 residues in exact agreement with the sequence predicted from the rabbit LTP cDNA (Table 3.2). Amino acid analysis of our protein also agreed well with the predicted composition based on the entire protein coding region of rabbit LTP cDNA (Table 3.3), also confirming the sample's identity as rabbit LTP. The specific activity of rabbit and human LTP were similar when assayed by radioisotopic CE transfer assay (3,377 versus 3,781 nmol h^{-1} mg^{-1}, respectively), as well as by pyrene-CE transfer assay (3.37 versus 2.71 $\text{min}^{-1}\mu\text{M}^{-1}$, respectively). Pure rabbit LTP could be isolated in 4 days using this procedure and activity was stable at 4° C for at least 3 months.

Rabbit plasma LTP behaved as a monomer of approximately 83 K during gel permeation chromatography (Fig. 3.4). Anomalous retention of human LTP has been observed during gel permeation and other forms of chromatography, possibly due to non-specific hydrophobic interaction with the gel matrix (Ohnishi *et al.*, 1990). Native isoelectric focusing of rabbit LTP revealed a ladder of isoforms (about 4) with pI ranging between 5.7 and 5.9 (Fig. 3.5). Focusing in the presence of 9 M urea, 2% Triton X-100, and mercaptoethanol resulted in LTP running mostly as a smear between pH 6.2 and 7.1, with a small amount running as a ladder at pI of 5.2 to 5.6 (not shown). This may have been due to aggregation of rabbit LTP or interaction with ampholytes under protein denaturing conditions.

3.3.3 Behavior of Rabbit LTP Towards Lipid Microemulsion Particles

The behavior of rabbit LTP towards lipid microemulsion particles consisting of a PC monolayer surrounding a TG core and approximating the size of LDL (Tajima *et al.*, 1983) was analyzed in terms of binding to these particles and its effect on protein conformation. Binding of LTP to lipid microemulsion particles was assessed by an ultracentrifugal binding assay. In this assay, when pure rabbit LTP, in the absence of lipid particles, is centrifuged according to the stated conditions, it becomes distributed in a 7 to 3 ratio between the bottom and top fractions, respectively, of the centrifuge tube (Fig. 3.6 panel A). When the LTP was combined with a 6- or 12-fold excess (by PC mass) of lipid microemulsion particles, following ultracentrifugation almost all the LTP is recovered in the top fraction (Fig. 3.6 panel B), apparently associated with floated lipid, which is 98% recovered in the top fraction. The amount of LTP recovered in the bottom fraction can be used as an estimate of the amount of free LTP in the top fraction. Accordingly, it should be 3/7 of the amount in the bottom fraction according to its relative distribution in the absence of lipid, and is thus very little. These results indicate that in assays of LTP mediated transfer of pyrene-neutral lipids between lipid microemulsion particles, which employed the same lipid particles as used here for binding studies, lipid transfer occurs while LTP is predominantly associated with the lipid particle surface.

Far-UV CD spectroscopy of pure rabbit LTP in buffered 150 mM NaCl gave a spectrum characteristic of a protein with a significant content of beta secondary structure (Fig. 3.7). Interpretation of the spectral data using the CONTIN program gave values of 0.30, 0.39, 0.12, and 0.19 for alpha-helix, beta-sheet, beta-turn, and remainder fractional content, respectively. For LTP in the presence of lipid microemulsion particles at similar amounts as in the binding studies above (approximately 7-fold excess by PC mass), very little change was seen in the CD spectrum (Fig. 3.7) or corresponding values of 0.26, 0.18, 0.25, and 0.31 computed for secondary structure fractional content in the order listed above. Thus, when rabbit LTP is placed in the presence of microemulsion, LTP becomes predominantly associated with these lipid particles, although this did not appear to be accompanied by major conformational alterations of the LTP polypeptide backbone.

3.4 Discussion

3.4.1 Purification of Rabbit LTP

For purification of rabbit LTP, hydrophobic interaction and cation exchange chromatography steps relied on the extreme hydrophobicity (Nagashima *et al.*, 1988) and acidic pI of rabbit LTP to achieve resolution from contaminating plasma proteins. The recovery of LTP activity from the Butyl-Toyopearl column was somewhat low, perhaps due to irreversible binding or gradual loss of protein during the lengthy column washings. Purification to apparent homogeneity by numerous criteria (SDS-PAGE and Coomassie Blue staining, N-terminal sequencing, and amino acid analysis) was attained with only 3,000 to 5,000-fold enrichment over plasma. This is in contrast with values of 28,000 to 108,000-fold reported in some more recent purifications of human LTP (Jarnagin *et al.*, 1987; Hesler *et al.*, 1987; Kato *et al.*, 1989; Ohnishi *et al.*, 1990). Rabbit plasma contains about 3-fold higher levels of LTP activity than humans (Ha and Barter, 1982), accounting partly for the lower enrichment necessary for purification of rabbit LTP. In a study from our lab, human LTP was purified about 40,000-fold relative to lipoprotein-depleted plasma (Ohnishi *et al.*, 1990); however, this is probably an overestimate of true purification since LTP activity of lipoprotein-depleted plasma is very low due to interference of the assay by something within the constituents used for lipoprotein precipitation. Another possibility is that although our protein is homogenous in composition, it may consist of a mixture of active and inactive forms, resulting in a low specific activity. However, simultaneous specific activity measurements of pure rabbit and human LTP (Ohnishi *et al.*, 1990) isolated within our lab were similar, and in contrast to LTP purified in other reports, the activity of our final preparation was extremely stable during storage.

3.4.2 Molecular Characteristics of Rabbit LTP

Similar to human LTP, the rabbit counterpart migrated during SDS-PAGE at M_r = 74,000, slower than expected for a protein of molecular mass 54,442 daltons, as predicted for LTP from its cDNA sequence (Nagashima *et al.*, 1988). Human LTP is a secreted glycoprotein (Swenson *et al.*, 1987) with four potential sites for Asn-linked glycosylation (Drayna *et al.*, 1987); 3 sites are always modified with carbohydrate and there is variable glycosylation at 1 site (Stevenson *et al.*, 1993). Rabbit LTP cDNA sequence contains 6 potential sites for Asn-linked glycosylation (Nagashima *et al.*, 1988) and the glycosylation pattern has not been examined further. Post-translational modification of human LTP by glycosylation has been shown to influence secretion as well as specific activity, suggesting that glycosylation is important for correct folding of LTP (Swenson *et al.*, 1987; Stevenson *et al.*, 1993). A smaller form of LTP can also arise from alternative splicing of LTP mRNA, resulting in deletion of exon 9 and formation of a shorter, inactive, and poorly secreted protein (Inazu *et al.*, 1992) which appears to be involved in regulation of secretion of active, full-length LTP from cells (Quinet *et al.*, 1993).

Rabbit LTP behaved as a monomer by gel permeation chromatography. Human LTP has been shown to behave similarly (Hesler *et al.*, 1987; Swenson *et al.*, 1988) whereas native gradient gel electrophoresis (Hesler *et al.*, 1987; Swenson *et al.*, 1989) and quasielastic light scattering (Takahashi *et al.*, 1993) have provided evidence for dimeric LTP quaternary structure under high concentrations, leading to suggestions of a lipid transfer mechanism involving dimeric LTP (Tall, 1993) as well as a possible role for heteromers of normal LTP and a form containing a missense mutation in regulation of LTP secretion from cells (Takahashi *et al.*, 1993; Quinet *et al.*, 1993). However, our lab has shown that human LTP behaves as a monomer at high concentrations by sedimentation equilibrium ultracentrugation (Ohnishi *et al.*, 1994) and the functional unit of rabbit LTP was previously shown to be monomeric by radiation inactivation (Ierides *et al.*, 1985). Finally, multiple pI isoforms of human plasma LTP have been reported (Hesler *et al.*, 1987, Jarnagin *et al.*, 1987; Kato *et al.*, 1989), with variable sialation accounting for many of the different isoforms (Kato *et al.*, 1989). Thus, the nature and function(s) of alternate forms and structures of LTP deserves further study.

3.4.3 Interaction of Rabbit LTP with Lipid Microemulsion Particles

Pure rabbit LTP interacted strongly with artificial lipid microemulsion particles previously shown to support LTP mediated transfer of pyrene-neutral lipids under activation by apolipoproteins (Milner *et al.*, 1991 and Chapter 2; Ohnishi and Yokoyama, 1993). The interaction between LTP and lipoprotein particles is an important regulatory factor since treatments such as increased LTP concentration (Morton, 1985) or addition of fatty acids (Sammett and Tall, 1985) or apoE (Kinoshita *et al.*, 1993) lead to increased LTP binding to lipoproteins and correspondingly in lipid transfer activity. The species of apoA-I or the resulting HDL formed is also important, as human LTP expressed in transgenic mice does not bind well to mouse HDL, while lipoprotein binding and lipid transfer activity increase when human apoA-I and LTP are co-expressed (Hayek *et al.*, 1992). The K_d of human LTP binding to lipid microemulsion particles was found to be approximately 10^{-8} M, about 10 times less than that for apoA-I and was unaltered when the lipid particle surface was saturated with apoA-I (Ohnishi *et al.*, 1994). Thus, the mechanism for apoA-I stimulation of LTP activity is unclear. One possibility is that although total LTP binding to microemulsion particles is unchanged by the presence of apoA-I, there is an increase in the particle off-rate during lipid transfer by a carrier mechanism. Alternatively, there could be greater transfer efficiency by LTP bound to the microemulsion surfaces in the presence of apoA-I during lipid transfer via a ternary complex mechanism.

Structural analysis of pure rabbit LTP by CD spectroscopy indicated a high content of beta structure and an alpha-helical content of about 30%, less than the 40% predicted from the protein sequence by Garnier, Osguthorpe, and Robson analysis (Nagashima *et al.*, 1988). The CD spectrum of human LTP is very similar to that of its rabbit counterpart (Ohnishi *et al.*, 1994). LTP is thought to have a highly stable tertiary structure since protease digestion does not cause inactivation (Hesler *et al.*, 1989). Human LTP has been predicted to contain only one short region of amphipathic helix which is at its C-terminal (residues 264-276 of human LTP), which is highly conserved in rabbit LTP (Drayna *et al.*, 1987; Nagashima *et al.*, 1988). The C-terminal region of the LTP molecule is known to be important for lipid transfer activity (Wang *et al.*, 1992) and the predicted helical region forms the epitope of an inhibitory mAb, as well as being involved in neutral lipid binding during the transfer process (Wang *et al.*, 1995).

Increased binding of LTP to lipoproteins occurs after binding of mAb Fab fragments to the C-terminal region of LTP (Swenson *et al.*, 1989). It is suggested that the Fab fragments mimick neutral lipid occupation of a binding site within LTP, resulting in a conformational change and enhancement of lipoprotein binding (Swenson *et al.*, 1989). No large scale changes in LTP secondary structure detectable by CD spectroscopy were found when LTP was bound to lipid microemulsion particles. However, subtle rearrangements in protein structure such as movement of a hinged region possibly involved in neutral lipid binding may not be detectable by our present study. Furthermore, activating apolipoproteins were not present in our binding study, so that while LTP was bound to the lipid particle surface, it may still not be in an active state. While binding of LTP to lipoproteins is thought to primarily involve ionic interactions with negative charges of the PL headgroups (Morton, 1985), the structural determinants of LTP involved in lipoprotein binding are uncertain. One possibility is a Lys triplet within a putative hydrophilic helix (residues 378-380 of human LTP; Wang *et al.*, 1991), corresponding to Gln-Lys-Lys in rabbit LTP (Nagashima *et al.*, 1988) , while another is the hydrophobic sequence beginning with Phe-Leu-Leu-Leu between residues 454 and 461 of human LTP (Au-Young and Fielding, 1992), corresponding closely to Cys-Leu-Leu-Leu, residues 474 to 481, of rabbit LTP (Nagashima *et al.*, 1988).

In summary, a simple and rapid procedure was developed for the isolation of rabbit LTP. The LTP thus isolated was molecularly characterized to verify its identity; as well, some biochemical properties of rabbit LTP in solution related to lipid transfer function were determined and contrasted with information obtained from studies of human LTP. Also, the behavior of rabbit LTP towards lipid microemulsion particles previously used to study apolipoprotein activation of LTP was studied. The microemulsion system seems well suited for further studies of the mechanism and regulation of LTP mediated neutral lipid transfer.

Table 3.1. Typical results for purification of rabbit LTP starting from 1.5 L of rabbit plasma.

Step	Protein (mg)	Activity (nmol/h)	Specific Activity (nmol/h/mg)	Purification (-fold)	Yield (%)
Plasma[a]	84,768	302,500	3.57	1.0	100
First Butyl-Toyopearl	497	61,547	123.8	34.7	20.3
CM-Toyopearl	219	42,167	192.5	54.0	13.9
Second Butyl-Toyopearl	0.69	8,680	12,580	3,534	2.9

[a] Data for lipoprotein-depleted plasma not given because activity was not measurable due to interference of the assay by this fraction.

Table 3.2. N-terminal amino acid sequencing of purified rabbit LTP.

Residue	1				5					10					15
Predicted[a]	Cys	Pro	Lys	Gly	Ala	Ser	Tyr	Glu	Ala	Gly	Ile	Val	Cys	Arg	Ile
Determined	X[b]	Pro	Lys	Gly	Ala	Ser	Tyr	Glu	Ala	Gly	Ile	Val	X	Arg	Ile

[a] Amino acid sequence of rabbit LTP predicted from its cDNA (Nagashima *et al.*, 1988).

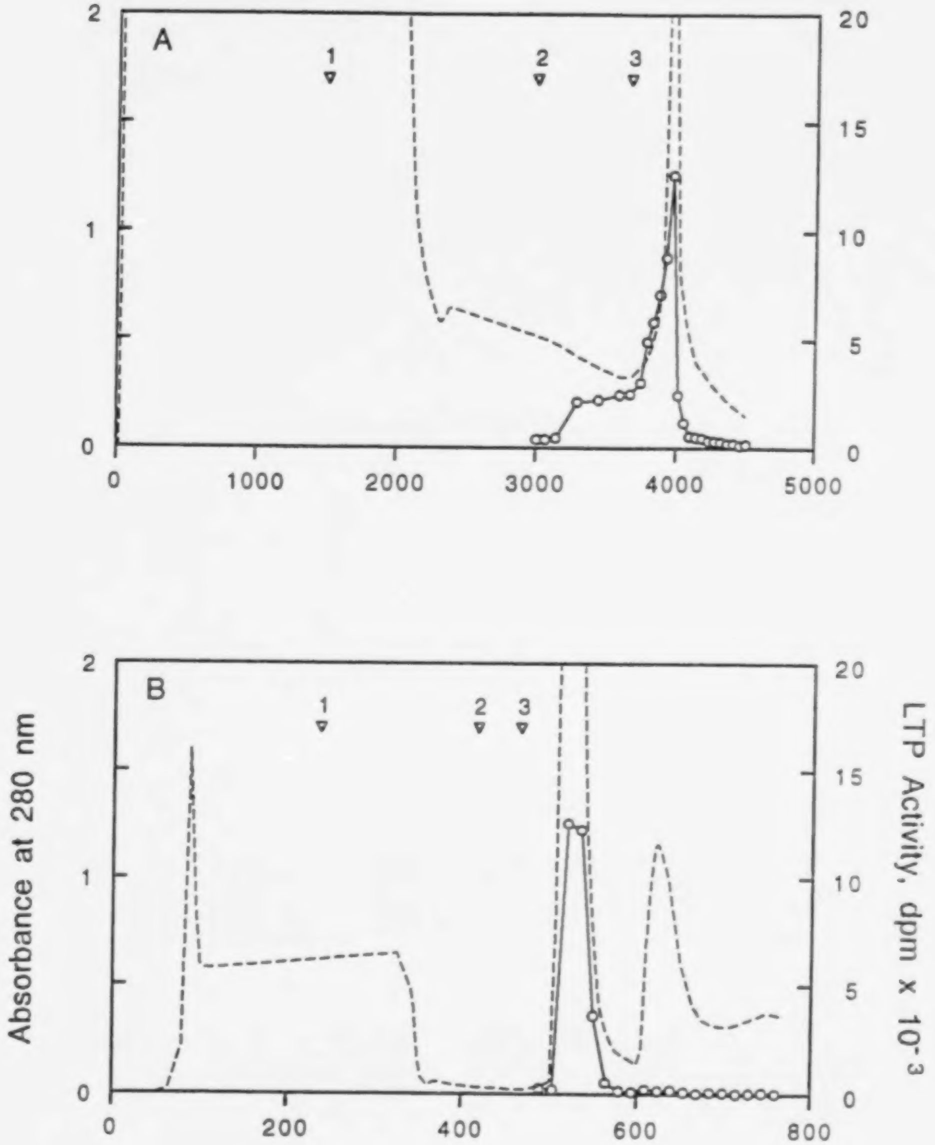
[b] X denotes cycles where no amino acid was identified by protein sequencing.

Table 3.3. Amino acid analysis of purified rabbit LTP[a]

Amino acid	Predicted content (mol/mol)	Predicted content (mol%)	Measured content[b] (mol%)
Asx	41	8.8	9.7
Thr	29	6.2	6.6
Ser	45	9.6	8.6
Glx	52	11.1	12.1
Gly	32	6.7	6.9
Ala	40	8.6	8.2
Val	41	8.8	7.6
Met	6	1.3	1.6
Ile	29	6.2	5.0
Leu	60	12.9	12.8
Tyr	10	2.1	4.0
Phe	27	5.8	5.3
His	11	2.4	2.3
Lys	21	4.5	4.6
Arg	23	4.9	4.7
Total	467	100	100

[a] Analysis performed on unmodified protein was unable to determine the number of Cys, Pro, or Trp, of which a total of 29 residues (9+17+3) are predicted for rabbit LTP (Nagashima *et al.*, 1988). Accordingly, the predicted relative content was calculated from 467 out of 496 residues for which data were obtained, allowing direct comparison of predicted and experimentally measured contents.

[b] Average of 2 trials.


A
B
1
2
3
Absorbance at 280 nm
LTP Activity, dpm x 10^{-3}
0
1
2
5
10
15
20
1000
2000
3000
4000
5000
200
400
600
800

(continued)

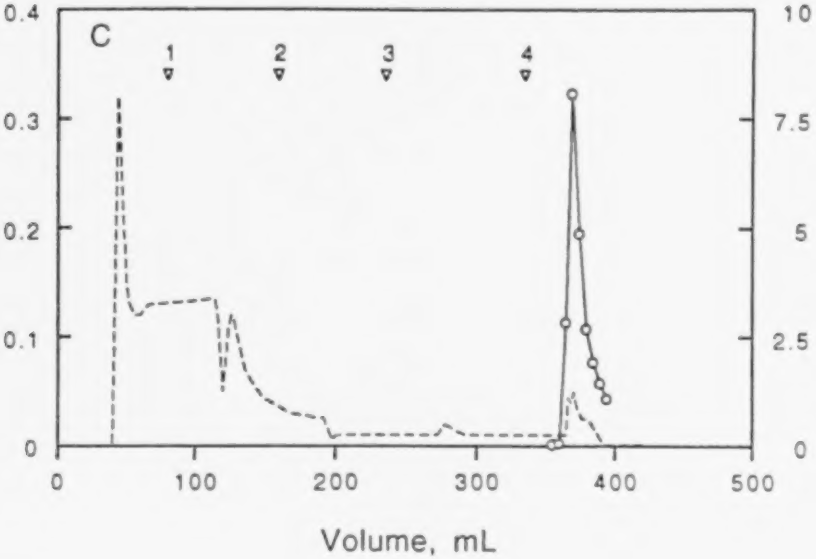


Figure 3.1. Column chromatography during purification of rabbit LTP. Panel A shows the elution from the first Butyl-Toyopearl column; panel B, elution from the CM-Toyopearl column; and panel C, elution from the second Butyl-Toyopearl column. The numbers indicate positions of buffer changes as described in the text (Experimental Procedures). The dashed line indicates UV absorbance, and circles represent LTP activity measurements by radioisotopic CE transfer assay.

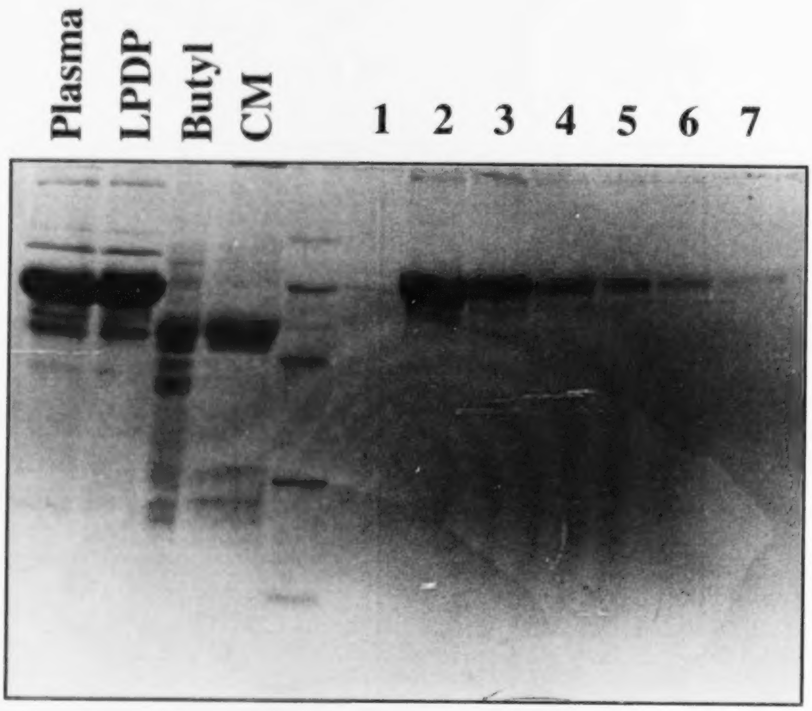


Figure 3.2. SDS-PAGE and Coomassie Blue staining of LTP fractions at different steps of purification. Lane marked Plasma contains 10 µg protein of rabbit plasma; LPDP contains 10 µg of lipoprotein-depleted plasma; Butyl contains 40 µg of pooled fraction after the first Butyl-Toyopearl column step; CM contains 40 µg of pooled fraction after the CM-Toyopearl step; and lanes marked 1 to 7 are 200 µL samples of individual fractions across the activity peak from the second Butyl-Toyopearl column, corresponding to 66, 8,077, 4,858, 2,670, 1,916, 1,432, and 1,080 dpm of transfer activity. The unmarked lane contains Bio-Rad molecular weight markers consisting of phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (42.7 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa).

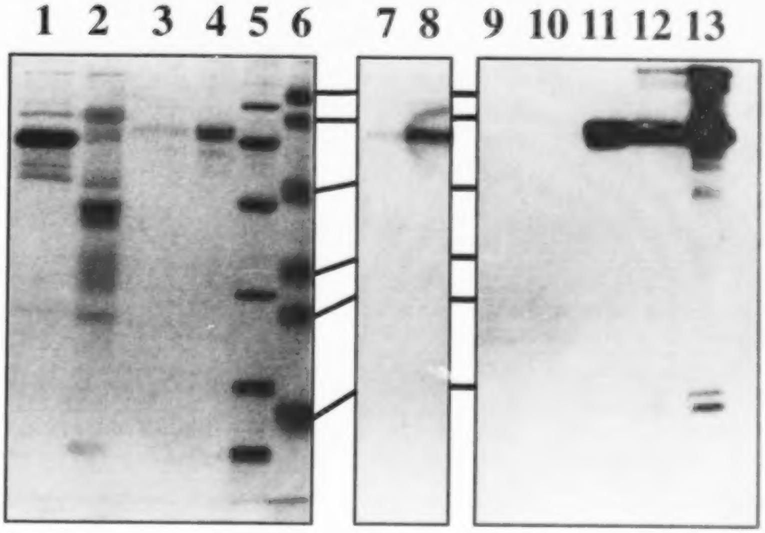


Figure 3.3. Immunostaining after SDS-PAGE of LTP fractions at different steps of purification. Lanes 1-6 depict Coomassie Blue staining of various fractions: lane 1 contains 0.05 μL rabbit plasma, 2 is the fraction after the first Butyl-Toyopearl column (4 μg), 3 and 4 are fractions after the second Butyl-Toyopearl column (0.2 and 0.9 μg, respectively). Lanes 7 and 8 are fluorographs after immunoblotting rabbit plasma (0.01 and 0.05 μL, respectively) using TP2 as the primary antibody (3 min exposure). Lanes 9 to 13 are a second fluorograph of the same membrane at shorter exposure (2 sec), with lanes 9 and 10 corresponding to 7 and 8 above; lane 11 contains the same material as lane 2, and lanes 12 and 13 contain the same as lanes 3 and 4, respectively. Lane 5 contains the same molecular weight markers as in Fig. 2. Lane 6 contains Bio-Rad prestained SDS-PAGE markers consisting of phosphorylase b (106 kDa), bovine serum albumin (80 kDa), ovalbumin (49.5 kDa), carbonic anhydrase (32.5 kDa), soybean trypsin inhibitor (27.5 kDa), and lysozyme (18.5 kDa). The angled lines depict the corresponding positions of the prestained markers on the membrane, which slant due to gel shrinkage.

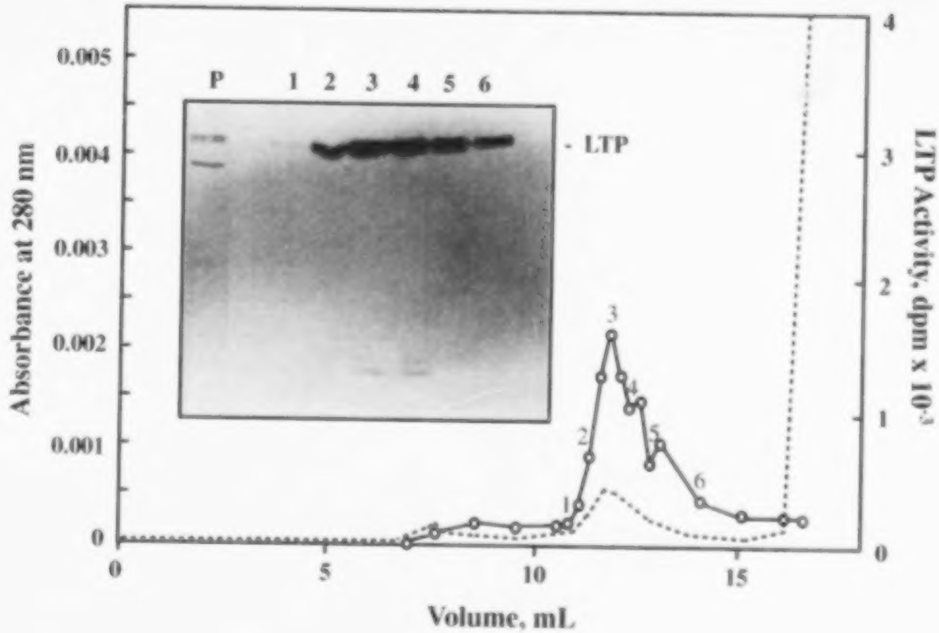


Figure 3.4. Superose 12 gel permeation chromatography of purified rabbit LTP. The dashed line indicates UV absorbance; the open circles represent LTP activity of the fraction. V_o was 7.4 mL by elution of dextran blue and V_t was 16.8 mL by elution of a copper salt. The inset shows a fluorograph after SDS-PAGE and TP2 immunoblotting of the numbered fractions in the elution profile. Lane P contains rabbit plasma (0.1 μL).

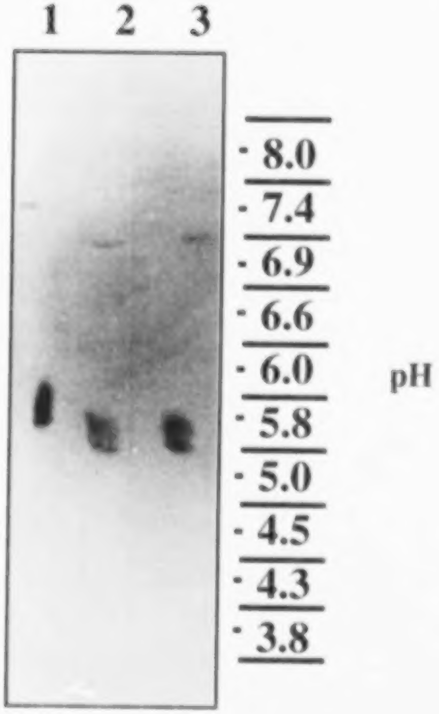

1
2
3
8.0
7.4
6.9
6.6
6.0
5.8
5.0
4.5
4.3
3.8
pH

Figure 3.5. Isoelectric focusing and TP2 immunoblotting of rabbit LTP. Lane 1 contains 1.2 μg purified LTP in native sample buffer; lanes 2 and 3 contain 1.2 μg purified LTP in native sample buffer plus 1% mercaptoethanol.

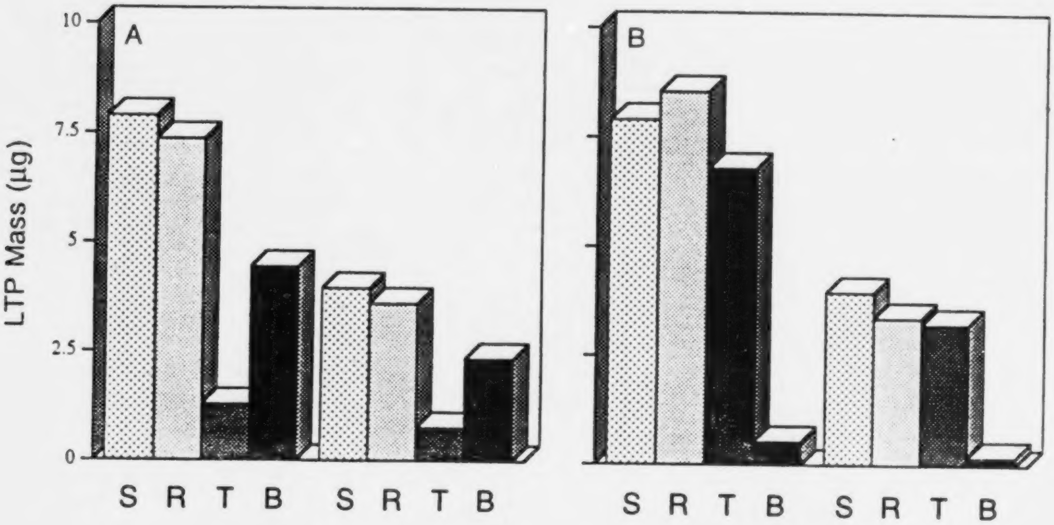


Figure 3.6. Binding of rabbit LTP to lipid microemulsion particles. Panel A depicts behavior of 2 concentrations of LTP following ultracentrifugation in the absence of microemulsion. Panel B shows behavior of same concentrations of LTP in the presence of microemulsion (65 µg by TG and 48 µg by PC). For each LTP concentration and condition, S, the starting amount of LTP, and R, the amount recovered after incubation, as well as T and B, the amount of LTP recovered in the top and bottom half fractions, respectively were measured.

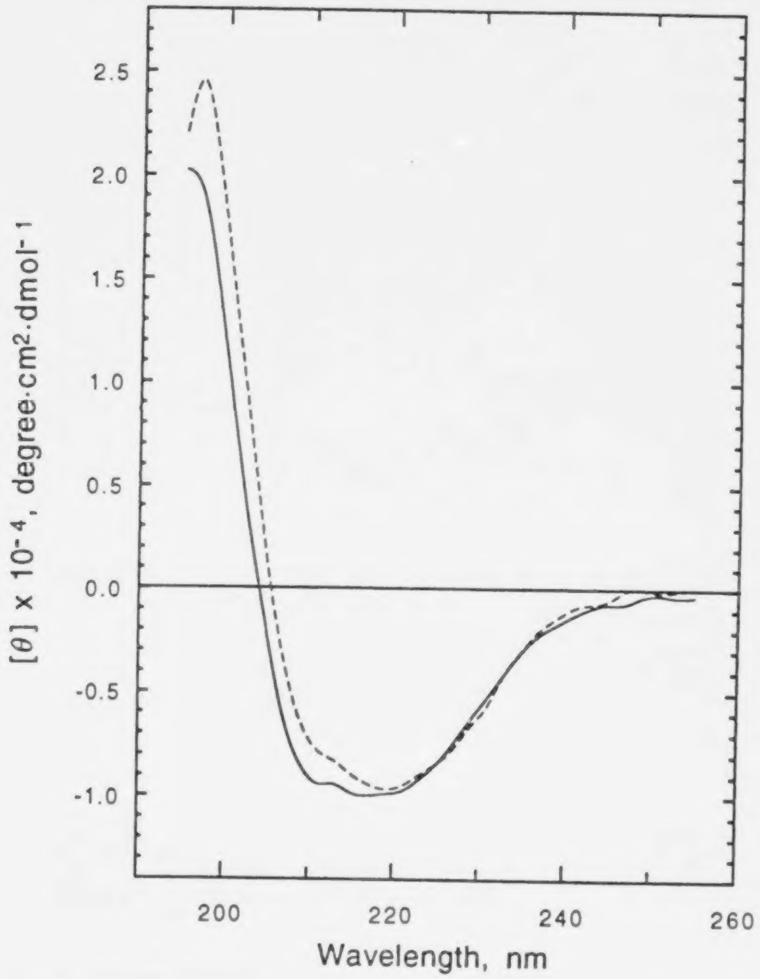


Figure 3.7. CD spectra of rabbit LTP taken at 25° C in 10 mM sodium phosphate (pH 7.4), 150 mM NaCl. The solid line is the spectrum of rabbit LTP alone (72 µg/mL), and the broken line is the spectrum of the same amount of LTP in the presence of lipid microemulsion particles (504 µg/mL by PC)

3.5 References

Au-Young, J., and Fielding, C.J. (1992) Synthesis and secretion of wild-type and mutant human plasma cholesteryl ester transfer protein in baculovirus-transfected insect cells: the carboxyl-terminal region is required for both lipoprotein binding and catalysis of transfer. *Proc. Natl. Acad. Sci. USA* 89, 4094-4098

Barter, P.J., and Jones, M.E. (1980) Kinetic studies of the transfer of esterified cholesterol between human plasma low and high density lipoproteins. *J. Lipid Res.* 21, 238-249

Drayna, D., Jarnagin, A.S., McLean, J., Henzel, W., Kohr, W. *et al.* (1987) Cloning and sequencing of human cholesteryl ester transfer protein cDNA. *Nature* 327, 632-634

Ha, Y.C., and Barter, P.J. (1982) Differences in plasma cholesteryl ester transfer activity in sixteen vertebrate species. *Comp. Biochem. Physiol.* 71B, 265-269

Hatch, F.T., and Lees, R.S. (1968) Practical methods for plasma lipoprotein analysis. *Adv. Lipid. Res.* 6, 1-68

Hayek, T., Chajek-Shaul, T., Walsh, A., Agellon, L.B., Moulin, P. *et al.* (1992) An interaction between the human cholesteryl ester transfer protein (CETP) and apolipoprotein A-I genes in transgenic mice results in profound CETP-mediated depression of high density lipoprotein cholesterol levels. *J. Clin. Invest.* 90, 505-510

Hesler, C.B., Swenson, T.L., and Tall, A.R. (1987) Purification and characterization of a human plasma cholesteryl ester transfer protein. *J. Biol. Chem.* 262, 2275-2282

Hesler, C.B., Tall, A.R., Swenson, T.L., Weech, P.K., Marcel, Y.L. *et al.* (1988) Monoclonal antibodies to the M_r 74,000 cholesteryl ester transfer protein neutralize all of the cholesteryl ester and triglyceride transfer activities in human plasma. *J. Biol. Chem.* 263, 5020-5023

Hesler, C.B., Brown, M.L., Feuer, D.S., Marcel, Y.L., Milne, R.W. *et al.* (1989) Structure-function analysis of plasma cholesteryl ester transfer by protease digestion and expression of cDNA fragments in *Escherichia coli*. *J. Biol. Chem.* 264, 11317-11325

Ierides, M., Dousset, N., Potier, M., Manent, J., Carton, M. *et al.* (1985) Cholesteryl ester transfer protein. Size of the functional unit determined by radiation inactivation. *FEBS Lett.* 193, 59-62

Ihm, J., Quinn, D.M., Busch, S.J., Chataing, B., and Harmony, J.A.K. (1982) Kinetics of plasma protein-catalyzed exchange of phosphatidylcholine and cholesteryl ester between plasma lipoproteins. *J. Lipid Res.* 23, 1328-1341

Inazu, A., Quinet, E.M., Wang, S., Brown, M.L., Stevenson, S. *et al.* (1992) Alternative splicing of the mRNA encoding the human cholesteryl ester transfer protein. *Biochemistry* 31, 2352-2358

Jarnagin, A.S., Kohr, W., and Fielding, C. (1987) Isolation and specificity of a M_r 74,000 cholesteryl ester transfer protein from human plasma. *Proc. Natl. Acad. Sci. USA* 84, 1854-1857

Kato, H., Nakanishi, T., Arai, H., Nishida, H.I., and Nishida, T. (1989) Purification, microheterogeneity, and stability of human lipid transfer protein. *J. Biol. Chem.* 264, 4082-4087

Kinoshita, M., Arai, H., Fukasawa, M., Watanabe, T., Tsukamoto, K. *et al.* (1993) Apolipoprotein E enhances lipid exchange between lipoproteins mediated by cholesteryl ester transfer protein. *J. Lipid Res.* 34, 261-268

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680-685

Lowry, O.L., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) Protein measurement with the folin phenol reagent. *J. Biol. Chem.* 193, 265-275

Marcel, Y.L., McPherson, R., Hogue, M., Czarnecka, H., Zawadski, Z. (1990) Distribution and concentration of cholesteryl ester transfer protein in plasma of normolipidemic subjects. *J. Clin. Invest.* 85, 10-17

Milner, T.G., Ko, K.W.S., Ohnishi, T., and Yokoyama, S. (1991) Enhancement of human lipid transfer protein reaction by apolipoprotein. *Biochim. Biophys. Acta* 1082, 71-78

Morton, R.E. (1985) Binding of plasma-derived lipid transfer protein to lipoprotein substrates. The role of binding in the lipid transfer process. *J. Biol. Chem.* 260, 12593-12599

Morton, R.E., and Zilversmit, D.B. (1983) Inter-relationship of lipids transferred by the lipid-transfer protein isolated from human lipoprotein-deficient plasma. *J. Biol. Chem.* 258, 11751-11757

Moulin, P., Cheung, M.C., Bruce, C., Zhong, S., Cocke, T. *et al.* (1994) Gender effects on the distribution of the cholesteryl ester transfer protein in apolipoprotein A-I-defined lipoprotein subpopulations. *J. Lipid Res.* 35, 793-802

Nagashima, M., McLean, J.W., and Lawn, R.M. (1988) Cloning and mRNA tissue distribution of rabbit cholesteryl ester transfer protein. *J. Lipid Res.* 29, 1643-1649

Nishida, H.I., Kato, H., and Nishida, T. (1990) Affinity of lipid transfer protein for lipid and lipoprotein particles as influenced by lecithin-cholesterol acyltransferase. *J. Biol. Chem.* 265, 4876-4883

Nishikawa, O., Yokoyama, S., Kurasawa, T., and Yamamoto, A. (1986) A method for direct incorporation of radiolabeled cholesteryl ester into human plasma low-density lipoproteins; preparation of tracer substrate for cholesteryl ester transfer reaction between lipoproteins. *J. Biochem.* 99, 295-301

Nishikawa, O., Yokoyama, S., Okabe, H., and Yamamoto, A. (1988) Enhancement of non-polar lipid transfer reaction through stabilization of substrate lipid particles with apolipoproteins. *J. Biochem.* 103, 188-194

Ohnishi, T., and Yokoyama, S. (1993) Activation of human lipid transfer protein by apolipoproteins. *Biochemistry* 32, 5029-5035

Ohnishi, T., Yokoyama, S., and Yamamoto, A. (1990) Rapid purification of human plasma lipid transfer protein. *J. Lipid Res.* 31, 397-406

Ohnishi, T., Hicks, L.D., Oikawa, K., Kay. C.M., and Yokoyama, S. (1994) Properties of human plasma lipid transfer protein in aqueous solution and at interfaces. *Biochemistry* 33, 6093-6099

Peterson, G.L. (1983) Determination of total protein. *Meth. Enzymol.* 91,95-121

Provencher, S.W., and Glöckner, J. (1981) Estimation of globular pprotein secondary structure from circular dichroism. *Biochemistry* 20, 33-37

Quinet, E., Yang, T.P., Marinos, C., and Tall, A. (1993) Inhibition of the cellular secretion of cholesteryl ester transfer protein by a variant protein formed by alternative splicing of mRNA. *J. Biol. Chem.* 268, 16891-16894

Sammett, D., and Tall, A. R. (1985) Mechanisms of enhancement of cholesteryl ester transfer protein activity by lipoplysis. *J. Biol. Chem.* 260, 6687-6697

Stevenson, S.C., Wang, S., Deng, L., and Tall, A.R. (1993) Human plasma cholesteryl ester transfer protein consists of a mixture of two forms reflecting variable glycosylation at asparagine 341. *Biochemistry* 32, 5121-5126

Swenson, T.L., Simmons, J.S., Hesler, C.B., Bisgaier, C., and Tall, A.R. (1987) Cholesteryl ester transfer protein is secreted by HepG2 cells and contains asparagine-linked carbohydrate and sialic acid. *J. Biol. Chem.* 262, 16271-16274

Swenson, T.L., Brocia, R.W., and Tall, A.R. (1988) Plasma cholesteryl ester transfer protein has binding sites for neutral lipids and phospholipids. *J. Biol. Chem.* 263, 5150-5157

Swenson, , T.L., Hesler, C.B., Brown, M.L., Quinet, E., Trotta, P.P. *et al.* (1989) Mechanism of cholesteryl ester transfer protein inhibition by a neutralizing monoclonal antibody and mapping of the monoclonal antibody epitope. *J. Biol. Chem.* 264, 14318-14326

Tajima, S., Yokoyama, S., and Yamamoto, A. (1983) Effect of lipid particle size on association of apolipoproteins with lipid. *J. Biol. Chem.* 258, 10073-10082

Takahashi, K., Jiang, X.-C., Sakai, N., Yamashita, S., Hirano, K. *et al.* (1993) A missense mutation in the cholesteryl ester transfer protein gene with possible dominant effects on plasma high density lipoproteins. *J. Clin. Invest.* 92, 2060-2064

Tall, A.R. (1993) Plasma cholesteryl ester transfer protein. *J. Lipid Res.* 34, 1255-1274

Tall, A.R., Granot, E., Brocia, R., Tabas, I., Hesler, C. *et al.* (1987) Accelerated transfer of cholesteryl esters in dyslipidemic plasma. Role of cholesteryl ester transfer protein. *J. Clin. Invest.* 79, 1217-1225

Wang, S., Deng, L., Brown, M.L., Agellon, L.B., and Tall, A.R. (1991) Structure-function studies of human cholesteryl ester transfer protein by linker insertion scanning mutagenesis. *Biochemistry* 30, 3484-3490

Wang, S., Deng, L., Milne, R.W., and Tall, A.R. (1992) Identification of a sequence within the C-terminal 26 amino acids of cholesteryl ester transfer protein responsible for binding a neutralizing monoclonal antibody and necessary for neutral lipid transfer activity. *J. Biol. Chem.* 267, 17487-17490

Wang, S., Kussie, P., Deng, L., and Tall, A. (1995) Defective binding of neutral lipids by a carboxyl-terminal deletion mutant of cholesteryl ester transfer protein. Evidence for a carboxyl-terminal cholesteryl ester binding site essential for neutral lipid transfer activity. *J. Biol. Chem.* 270, 612-618

Weinberg, R.B., Cook, V.R., Jones, J.J., Kussie, P., and Tall, A.R. (1994) Interfacial properties of recombinant human cholesterol ester transfer protein. *J. Biol. Chem.* 269, 29588-29591

Yokoyama, S., Hayashi, R., Kikkawa, T., Tani, N., Takada, S. *et al.* (1984) Specific sorbent of apolipoprotein B-containing lipoproteins for plasmapheresis. Characterization and experimental use in hypercholesterolemic rabbits. *Arterioscl.* 4, 276-282

CHAPTER 4

Generation of Novel Inhibitory mAbs Against Rabbit LTP and Characterization of Their Different Inhibitory Mechanisms[1]

4.1 Introduction

The amino acid sequence of LTP bears similarity to two other mammalian proteins, LPS binding protein (LBP) and bactericidal permeability increasing protein (BPI), suggesting that it evolved from an ancient family of lipid binding proteins (Drayna *et al.*, 1987). These last two proteins are important in regulating host responses to Gram-negative bacterial infections. LBP is an acute phase protein which acts as a soluble receptor for LPS (Tobias *et al.*, 1986), recognizing the lipid A moiety (Tobias *et al.*, 1989). The complex thus formed binds to CD14 of monocytic cells, thereby eliciting a cytokine response, most importantly TNF secretion, leading to endotoxemic shock (Wright *et al.*, 1990; Gallay *et al.*, 1993). BPI is a neutrophil granule protein which also binds LPS and abrogates the host response to endotoxin through inhibition of IL-6 production (Jin *et al.*, 1995), as well directly killing Gram-negative bacteria. The 25 kDa N-terminal domain of BPI is rich in basic and hydrophilic residues (Gray *et al.*, 1989), and possesses all the antibacterial (Ooi *et al.*, 1987) and much of the endotoxin neutralizing (Ooi *et al.*, 1991) activity of the 50-60 kDa parent protein, while the function of the more acidic, hydrophobic C-terminal is unclear and may serve as a membrane anchor (Gray *et al.*, 1989). LBP and BPI share about 40% amino acid identity (Gray *et al.*, 1989) suggesting that these proteins employ similar protein structural determinants for LPS binding.

The structural determinants contributing to the activity of LTP are less well defined. The most well characterized determinant is the C-terminal region which contains the epitope of an inhibitory mAb, TP2, as well as being essential for binding and transfer of both CE and TG (Wang *et al.*, 1992). On the other hand, results from dissociating agents which influence transfer of one neutral lipid to a greater extent than the other, including sulfhydryl

[1] A version of this chapter has been published: Ko, K.W.S., Ohnishi, T., and Yokoyama, S. (1994) *J. Biol. Chem.* 269, 28206-28213.

reagents such as pCMPS (Hopkins and Barter, 1980; Morton and Zilversmit, 1982), mAbs (Fukasawa *et al.*, 1992; Melchior *et al.*, 1995), and inhibitory cholesterol analogs (Busch and Harmony, 1990; Bisgaier *et al.*, 1994; Epps *et al.*, 1995; Melchior *et al.*, 1995), have prompted the suggestion of independent sites for CE and TG transfer. PL transfer activity seems to be independent of neutral lipid transfer activity based on studies by activity measurements (Morton and Zilversmit, 1983) and the generation of recombinant LTP variants that are defective in neutral lipid transfer but have normal PL transfer activity (Wang *et al.*, 1992). Another crucial step for lipid transfer activity is interaction of LTP with the lipoprotein surface. Deletion of a region near the C-terminal of LTP was demonstrated to abolish lipoprotein binding (Au-Young and Fielding, 1992), while a region containing a Lys triplet (Wang *et al.*, 1991) has also been suggested to participate in this role.

In this study, mAbs have been developed for further investigating the mechanism of neutral lipid transfer between lipoproteins by LTP. Three mAbs recognizing LTP and inhibiting transfer activity were obtained. From their effects on LTP activity in various assay systems, it was concluded that our novel mAbs are targeted against two different determinants of LTP activity: (1) a region involved in neutral lipid binding and transfer; and (2) a region involved in lipoprotein binding. Our results also provide support for a heteroexchange mechanism for net transfer of neutral lipids involving a single site where CE and TG compete for binding and transfer, rather than independent sites within LTP for CE and TG transfer.

4.2 Experimental Procedures

4.2.1 Immunizations

Female 6 week old Balb/C mice (University of Alberta Lab Animal Services) were immunized with native purified rabbit LTP (approximately 3 μg) isolated as previously described (Ko *et al.*, 1993 and Chapter 3) and combined with MPL+TDM adjuvant (RIBI Immunochem), by intraperitoneal injection. At week 3, a secondary injection (3 μg LTP) was given and after detection of positive, inhibitory activity towards rabbit LTP mediated radioisotopic CE transfer (below) in tailbleed serum (control serum was negative) at week 4, mice were boosted again at week 6. At week 12, final boosting intraperitoneally and intravenously (3 μg LTP with and without adjuvant, respectively) was performed before spleen cell fusion.

4.2.2 Fusions and mAb Production

Mouse myeloma cell line P3X63 Ag8 (kindly supplied by Dr. Tim Mosmann, Department of Immunology, University of Alberta) was cultured in DMEM, 8% FCS, and 50 μg/mL gentamycin (all from Gibco) (Myeloma medium) at 37° C in a CO_2 (7%) incubator, splitting at 1:40. On the day of the fusion, 10^8 cells were collected (1,500 rpm for 10 min, benchtop centrifuge) in RPMI, 5% FCS, 50 mM 2-mercaptoethanol (Sigma), and 50 μg/mL gentamycin (Rinse medium). The mouse was killed by cervical dislocation and the spleen was excised aseptically, placed into a petri dish, and washed with prewarmed Rinse medium. The spleen was then homogenized in 5 mL Rinse medium using 2 sterilized, frosted microscope slides, and splenocytes were collected and resuspended in 10 mL Rinse medium. The splenocytes and myeloma cells were combined in a sterile centrifuge tube (Corning), and the volume was adjusted to 50 mL with RPMI (Gibco) alone. The cells were collected and washed once more with RPMI before drying the pellet carefully. Then 1 mL prewarmed 50% PEG (Sigma) was added with gentle shaking, followed by 1 min standing. Then 500 mL RPMI was added over 45 sec, followed by 500 mL over 15 sec, 5 mL over 5 min and 15 mL over 5 min. One mL FCS and 25 mL RPMI, 10% FCS, 50 mM 2-mercaptoethanol, 50 μg/ml gentamycin, 1X HAT (Sigma) (HAT medium) were then added and cell density was adjusted to 2 x 10^6 fused cells/mL with further HAT medium before aliquoting 100 μL/well into sterile 96 well plates (Corning). Next day, 150 μL HAT medium was added; subsequently, media changes of 170 μL were performed twice a week for 2 weeks with HAT medium, followed by subculturing and expansion in HT medium (HAT medium, substituting HT (Sigma) for HAT) for 4 weeks, during which preliminary screening by inhibition assay or dot blotting (below) was performed. Positive wells were cloned by limiting dilution at least three times with a splenocyte feeder cell method. Large amounts of individual mAbs were produced by intraperitoneal injection of clonal hybridomas in phosphate-buffered saline (3 x 10^6 cells) into pristane (Sigma) primed mice and harvesting of serum and ascites fluid 1-2 weeks later. Antibodies were purified by 25-50% ammonium sulfate precipitation and

Protein A Sepharose 4 (Pharmacia) chromatography under the high salt condition (Harlow and Lane, 1988).

4.2.3 Inhibition Assay Screening and Immunotitration Studies

Inhibition assay screening and immunotitration studies evaluated the ability of mAb to inhibit radioisotopic CE or TG transfer mediated by rabbit or human lipoprotein-deficient plasma (LPDP) as an LTP source. Rabbit and human LPDP were prepared by *d* = 1.25 NaBr ultracentrifugation of fresh human or previously frozen rabbit (Pelfreez, Rogers, AR) plasma at 50,000 rpm (184,000 x *g*) for 60 h (Beckman 70 Ti) followed by removal of floated lipoproteins and dialysis of the infranatant with LTP buffer, 1 mM EDTA (Ko *et al.*, 1993 and Chapter 3). For screening of tailbleed serum and hybridoma culture supernatants, 25 µL rabbit LPDP was pretreated with diluted sera or supernatant for 2 h at 4° C before subjecting the entire mixture to a radioisotopic CE transfer assay (Ko *et al.*, 1993 and Chapter 3). Wells secreting antibodies inhibitory towards radioisotopic CE transfer by LTP were cloned by limiting dilution, and 2 clones: 2-8G and 3-9F, were selected for further study.

For the immunotitration studies, various amounts of the IgG fraction were preincubated with 25 µL rabbit or 50 µL human LPDP for 2 h at 4° C before performance of a radioisotopic CE (above) or TG transfer assay on the entire mixture. For the TG transfer assay, donor radioisotopic TG-labeled LDL was prepared using the method described for making [^{3}H]cholesteryl oleate-labeled LDL (Ko *et al.*, 1993 and Chapter 3), except substituting vesicles prepared with 0.5 mCi [^{3}H]glycerol trioleate (Amersham) and 4.5 mg PC (Avanti Polar Lipids) in the method. Final TG concentration and specific radioactivity of radiolabeled LDL thus obtained was 1.27 mM and 26,600 dpm/nmol, and radioisotopic TG transfer assays contained 10 µg TG in LDL and the same amount of acceptor HDL (40 µg cholesterol, and 3.7 µg TG) as for the radioisotopic CE transfer assays.

4.2.4 Dot Blot Screening

Hybridoma culture supernatants (2 µL) were spotted onto nitrocellulose membranes (Bio-Rad) which had been preabsorbed with pure native rabbit LTP (by soaking in 1-5 µg LTP/cm^2 for 1 h), and then blocked with TBS, 5% milk (Ko *et al.*, 1993 and Chapter 3). Adherent antibodies were detected by treatment of the membrane with horseradish peroxidase-linked sheep anti-mouse Ig (Amersham) and fluorography (Ko *et al.*, 1993 and Chapter 3). Wells secreting antibodies recognizing LTP were cloned by limiting dilution using the dot blot screen, and clone 14-8H was selected for further study.

4.2.5 Pyrene-Neutral Lipid Transfer Assays

The apolipoprotein-activated transfer of pyrene-CE from a donor to a 9-fold excess of acceptor lipid microemulsion particles was measured as previously described (Ohnishi and Yokoyama, 1993). For analysis of pyrene-TG transfer, pyrene-TG labeled donor microemulsion had to be prepared using the procedure outlined (Ohnishi and Yokoyama, 1993) but substituting the same weight of 1,2 dioleoyl-3-(pyren-1-yl) decanoyl-*rac*-glycerol (Sigma) for pyrene-CE. Acceptor microemulsions were the same in both cases. When the mAbs were tested, they were preincubated with pure rabbit or human LTP for at least 30 min at 4° C before performing the assay. Purified rabbit (Ko *et al.*, 1993 and Chapter 3) and human (Ohnishi *et al.*, 1990) LTP for the assays were prepared as described.

4.2.6 Gel Electrophoreses and Immunoblotting

SDS-PAGE was performed as previously described (Ko *et al.*, 1993 and Chapter 3). Native-PAGE was performed using the same mini-gel apparatus and a 4% acrylamide stacking/8% acrylamide separating gel run in 89 mM Tris-HCl (pH 8.4), 89 mM boric acid, and 2 mM EDTA (TBE) at 120 V constant voltage for 1 h. Native sample buffer was 2X TBE, 20% glycerol, and 0.02% bromophenol blue and samples were mixed 1:1 (v/v) before loading directly to the gel. Immunoblotting was performed as previously described (Ko *et al.*, 1993 and Chapter 3), substituting various mAbs as the primary antibody.

4.2.7 Lipid Microemulsion Particle Binding Analysis

The effects of mAb treatment on LTP binding to lipid microemulsion particles were assessed by preincubating 2 µg rabbit or human LTP with 20 µg of various IgG fractions for 30 min at 4° C before addition of LTP buffer, 0.5% ovalbumin (Sigma) with or without microemulsion (100 µg in TG and and 70 µg in PC) in a total volume of 200 µL and incubation at 37° C for 15 min. The samples were then centrifuged and separated as previously described (Ko *et al.*, 1993 and Chapter 3) before 10 µL aliquots were analyzed by SDS-PAGE and immunoblotting as described above, using mAb 14-8H (recognizes native and denatured rabbit and human LTP) as the primary antibody (30 μg IgG/mL) to immunostain LTP in all fractions.

4.2.8 Human Plasma Incubation Studies

Plasma (16 mL) was obtained from 6 healthy, overnight fasted subjects (3 male, 3 female) by collection of blood into 0.1% EDTA and centrifugation at 4° C (Ko *et al.*, 1993 and Chapter 3). Care was taken to cool the blood as rapidly as possible, with immersion of the catheter tubing in ice water during blood collection. Half of each plasma sample was treated with 2 mM of 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) to inhibit LCAT, the other half was left untreated. An aliquot (2 mL) of each was removed and kept at 4° C, a temperature where LTP (and LCAT) are inactive, to serve later as a control. The remaining 6 mL of DTNB treated or untreated plasma were divided into 3 fractions each for incubation at 37° C for 24 h, one without further treatment, and the other 2 in presence of either mAb 3-9F or 14-8H (64 μg IgG/mL). After completion of incubation, all tubes were placed on ice and VLDL (0.25 mL) was removed after ultracentrifugal flotation (d = 1.006 g/mL) at 100,000 rpm (412,000 x g) for 2 h at 4° C (Beckman TLA-100.3). HDL was obtained by heparin (250 U/mL)/$MnCl_2$ (0.09 M) treatment of an aliquot of the d > 1.006 g/mL fraction (Tall *et al.*, 1987). Total cholesterol, free cholesterol, and TG content were determined for VLDL, d > 1.006 fraction, and HDL, using enzymatic lipid assay kits from Wako (free cholesterol and TG) and Sigma (total cholesterol) and values for LDL lipids were determined by subtraction of HDL values from d > 1.006 g/mL values. Interference of lipid assay readings by DTNB treatment were corrected by linear regression (untreated, control plasma samples versus matching DTNB-treated, control plasma samples) and correction of the readings for all DTNB treated, 37° C incubated samples according to the linear equation obtained. CE values for each lipoprotein fraction were calculated from difference of free from total cholesterol values, reported as mg/dL of cholesterol moiety. TG values have not been corrected for free glycerol, which should show up mainly in the HDL and total TG values, leading to an overestimate of about about 10 mg/dL in each case. Statistical analysis of the data was performed by paired t test (two-tailed). The mean (SEM) plasma lipid values for the entire group were total cholesterol 205.8 (12.1) mg/dL, HDL cholesterol 48.0 (3.6) mg/dL, and triglyceride 87.5 (14.8) mg/dL.

In other studies with human plasma, samples were treated with the same amounts of DTNB or mAb as above, before subjecting 50 μL aliquots to radioisotopic CE and TG transfer assays. In another study, [^{3}H]cholesteryl oleate or [^{3}H]triolein labeled HDL were prepared by ultracentrifugal flotation in NaBr (d < 1.21 g/mL) of flow through material from the dextran sulfate-cellulose column during preparation of radiolabeled LDL described above. Radiolabeled HDL (30 μg total cholesterol) was added to 0.5 mL human plasma, untreated or pretreated with mAb as above, thus increasing the HDL cholesterol by about 10%. Incubation at 37° C was performed, with 150 μL aliquots removed at various time points and placed on ice before total and HDL radioactivity of the samples were determined. HDL radioactivity was determined in the supernatant after heparin/$MnCl_2$ treatment of the plasma samples as described above, and lower density lipoprotein radiaoactivity was the difference between total and HDL counts.

4.2.9 Analytical Methods

Protein was determined by Lowry assay (Lowry *et al.*, 1951), with BSA (bovine serum albumin) (Pierce) as a standard, except for purified LTP, where it was determined by BCA assay (Pierce) with BSA as a standard. Antibody class and subclass determination was performed with an mAb Isotyping Kit (Pierce) using their antigen-independent screening protocol.

4.2.10 Other Materials

TP2 mAb was a generous gift from Drs. Yves Marcel and Ross Milne (University of Ottawa Heart Institute).

4.3 Results

4.3.1 Raising of mAbs Against Rabbit LTP

mAbs against rabbit LTP were raised by immunization of mice with purified protein. Hybridoma culture supernatants were initially screened by two methods in order to detect presence of antibodies against LTP. Firstly, supernatants were directly screened for ability to inhibit radioisotopic CE transfer between lipoproteins stimulated by rabbit LPDP as an LTP source. Several inhibitory clones were detected and clones 2-8G and 3-9F were selected for further study. Additionally, the supernatants were screened by dot blotting, as another method to detect antibodies recognizing rabbit LTP, and among several clones 14-8H was selected for further study. Supernatant of 14-8H was weakly inhibitory when screened for inhibition of LTP mediated radioisotopic CE transfer. Surprisingly, the supernatant antibodies from the inhibitory clones bound only weakly in the dot blotting screen. Isotyping of the supernatants revealed that all three hybridoma clones secreted mAbs of the IgG_1 subclass with *k* light chains. Subsequently, the clones were used for production of mouse ascites fluid from which the IgG fraction was purified and used for the following studies.

4.3.2 Effects of mAbs on Radioisotopic Neutral Lipid Transfer

Immunotitration of radioisotopic CE and TG transfer from LDL to HDL stimulated by rabbit or human LPDP was carried out. In agreement with the initial results (above), mAbs 2-8G and 3-9F, obtained by direct inhibition screening, both inhibited rabbit LTP mediated CE transfer in a dose dependant manner whereas 14-8H, obtained by dot blotting and weakly inhibitory, had little effect on CE transfer (Fig. 4.1, panel A). While 2-8G inhibited rabbit LTP, it was unable to inhibit CE transfer mediated by human LTP (Fig. 4.1, panel B). When radioisotopic TG transfer was analyzed, in addition to 2-8G and 3-9F, 14-8H was also able to inhibit rabbit LTP in a dose dependant manner (Fig. 4.2, panel A). Once again, 2-8G had no effect on human LTP whereas 3-9F and 14-8H did cross react (Fig. 4.2, panel B). Thus, we have obtained 3 different mAbs: 2-8G, which neutralizes radioisotopic neutral lipid transfer, inhibiting both CE and TG transfer, and is specific for rabbit LTP; 3-9F, which neutralizes radioisotopic neutral lipid transfer by rabbit LTP and cross reacts with human LTP; and 14-8H, which dissociates radioisotopic neutral lipid transfer, inhibiting TG transfer to greater extent, and as well, it cross reacts with human LTP. Dissociation of neutral lipid transfer by various treatments has lead several groups to suggest the existence of independent sites for CE and TG transfer within LTP (see Introduction). Alternatively, dissociation of CE and TG transfer may occur when the larger transfer substrate, TG, is hindered from entering a single neutral lipid binding site involved in lipid transfer, as occurs during TP2 Fab fragment treatment of human LTP (Swenson *et al.*, 1989). The mechanisms for the different effects of our mAbs, neutralization and dissociation of radioisotopic neutral lipid transfer by LTP, were further investigated.

4.3.3 *Effects of mAbs on Pyrene-Neutral Lipid Transfer*

The effects of neutralizing and dissociating mAb on LTP mediated pyrene-neutral lipid transfer between lipid microemulsion particles were examined. Within this system, the LTP reaction has been previously shown to be activated by the presence of exchangeable, amphiphilic alpha-helical apolipoproteins (Ohnishi and Yokoyama, 1993). For a negative control, the assay was performed in the absence of LTP, with apoA-I alone, and maximum activity was obtained with LTP and apoA-I for the positive control (Table 4.1). In this assay, 2-8G and 3-9F, which neutralized radioisotopic neutral lipid transfer, also blocked both pyrene-CE and -TG transfer (Table 4.1). Surprisingly, 14-8H, which dissociated radioisotopic neutral lipid transfer, affecting [^{3}H]CE transfer less, inhibited pyrene-CE as well as -TG transfer mediated by rabbit LTP (Table 4.1). When human LTP was substituted for rabbit LTP, once again, 2-8G was ineffective in inhibiting either pyrene-CE or -TG transfer mediated by human LTP whereas 3-9F and 14-8H blocked transfer of both (Table 4.1). Thus, there was consistency in the effects of 2-8G and 3-9F, which both neutralize both radioisotopic and pyrene-neutral lipid transfer, while there was inconsistency in the effect of 14-8H, which dissociates radioisotopic neutral lipid transfer but neutralizes pyrene-neutral lipid transfer. One possible explanation for these results according to the model of LTP having a single neutral lipid binding and transfer site is that 14-8H blocks entry of both pyrene-neutral lipids to this site in the pyrene assay, perhaps because of the added bulk of the pyrene moiety. Meanwhile, in the radioisotopic assay, 14-8H only blocks entry of the larger transfer substrate, TG, similar to the action of TP2 Fab fragment (Swenson *et al.*, 1989). The pyrene-lipid assay system did not provide further information about mechanisms for inhibition of LTP activity by 2-8G and 3-9F.

4.3.4 *Effects of mAbs on LTP Binding to Lipid Microemulsion Particles*

We previously established conditions for analyzing LTP binding to lipid microemulsion particles employing an ultracentifugation assay (Ko *et al.*, 1993 and Chapter 3). Within this binding assay, LTP distribution following ultracentrifugation varied depending on the absence or presence of microemulsion. In the absence of microemulsion, rabbit LTP mass, as reflected by LTP activity measurement, became distributed 70%:30% between bottom and top half tube fractions after ultracentrifugation whereas in the presence of microemulsion, the LTP became almost entirely associated with the top half tube fraction, which contained all the floated microemulsion as well. To examine the effects of our mAbs on LTP binding to lipid microemulsion particles, LTP activity measurement could not be employed to analyze distribution, due to inhibition of lipid transfer activity by antibody. Instead, SDS-PAGE and immunoblotting were used to assess LTP content of the two tube fractions after performing the binding assay in the presence of each mAb. Consistent with the results by LTP activity measurements, a major portion of rabbit or human LTP was recovered in the bottom half tube fraction when centrifuged in the absence of microemulsion (Fig. 4.3, leftmost column). In the presence of microemulsion, LTP becomes almost entirely recovered in the top half fraction after centrifugation (Fig. 4.3, second from left column) When rabbit LTP is pretreated with 2-8G before centrifugation in the presence of lipid microemulsions, there is an appreciable increase in amount of LTP in the bottom half fraction compared to when mAb is absent, indicating that this rabbit specific mAb interferes incompletely with binding of rabbit LTP to the lipid particles (Fig. 4.3, panel A center column). On the other hand, with human LTP, 2-8G had no effect on the binding of LTP to lipid microemulsions (Fig. 4.3, panel B center column), consistent with its species-specific inhibition of rabbit LTP in radioisotopic and pyrene-neutral lipid transfer inhibition assays. mAb 3-9F appeared to completely block binding of both rabbit or human LTP to microemulsion (Fig. 4.3, second from right column), resulting in an LTP distribution similar to that of LTP in the absence of microemulsion. On the other hand, 14-8H had no effect on binding of LTP to lipid microemulsions (Fig. 4.3 rightmost column), as the distribution was similar to untreated LTP in the presence of microemulsion, where LTP becomes predominantly associated with the top half fraction. Following all centrifugations of mAb treated LTP in the presence of microemulsion, greater than 97% of the lipid present (by TG mass) was recovered in the top half fraction. Thus, 2-8G and 3-9F, which both neutralize both radioisotopic and pyrene-neutral lipid transfer by LTP, both diminish LTP binding to lipid microemulsion particles, suggesting that they interfere with a region of LTP involved in lipoprotein binding. The results from the pyrene-neutral lipid transfer studies implied that 14-8H may recognize a site involved in transfer substrate binding, which is consistent with a lack of effect of 14-8H on lipoprotein or lipid microemulsion particle binding by LTP.

4.3.5 *Effects of mAbs on Neutral Lipid Transfer Within Incubated Human Plasma*

mAbs 3-9F and 14-8H, which neutralize and dissociate radioisotopic neutral lipid transfer, respectively, and both cross react with human LTP, were investigated for effects on radioisotopic and mass transfer of neutral lipid within incubated human plasma. mAb 3-9F treatment of human plasma totally inhibited its ability to stimulate radioisotopic CE and TG transfer from LDL to HDL in our standard assay, and 14-8H completely inhibited TG transfer while decreasing CE transfer roughly 30% (Fig. 4.4). Also, DTNB treatment of human plasma, to inhibit LCAT by modification of Ser residues, did not alter either CE or TG transfer activity of LTP in this assay. Radioisotopic transfer of neutral lipids between endogenous lipoproteins during incubation of human plasma was examined by spiking the plasma with radiolabeled HDL, and 3-9F completely prevented CE and TG label from being chased to lower density lipoproteins, while 14-8H completely blocked TG transfer and inhibited CE transfer about 45% (Fig. 4.5). Thus, 3-9F treatment blocked all radioisotopic neutral lipid transfer under these conditions while 14-8H permitted residual CE transfer activity.

Incubation of human plasma pretreated with DTNB at 37° C for 24 h demonstrated net CE and TG mass transfer among lipoprotein classes (Table 4.2). Compared to control plasma samples kept at 4° C, after the incubation period, HDL CE decreased in the samples incubated at 37° C, with accompanying increases in VLDL and LDL CE. The net increases are greater than the decrease in HDL CE, and may be overestimates as suggested by the larger errors associated with the VLDL and LDL CE measurements. The higher errors in LDL CE values for all the conditions is unexplained, but occurred in a similar study (Yen *et al.*, 1989) and would appear to account well for the unexpected increase in total CE values for all 3 incubated groups. However, a lack of change in free/total cholesterol ratios in all the groups indicates that LCAT was effectively inhibited in this study, so that changes in lipoprotein CE mass should be attributable to lipid transfer (and error). Decreased VLDL TG following 37° C incubation, on the other hand, was well compensated for by accompanying increases in LDL and HDL TG levels.

Thus, 37° C incubation of human plasma with LCAT inhibited leads to net mass transfer of CE from HDL to VLDL and perhaps LDL, with TG moving from VLDL to LDL and HDL. An overall net increase of core neutral lipid content may be observed in LDL, which gained TG and possibly CE, but not in VLDL or HDL, where an increase in the content of one neutral lipid appeared to be fairly well compensated by an approximately equimolar decrease in the other one (Table 4.3).

The effects of radioisotopic neutral lipid transfer neutralizing and dissociating mAbs on neutral lipid net mass transfer in incubated human plasma with LCAT inactivated were examined. mAb 3-9F or 14-8H treatment effectively abolished all the changes in lipoprotein neutral lipid content normally seen following incubation of LCAT-inactivated plasma at 37° C, including the increase in LDL total neutral lipid content (Table 4.2). Thus, all neutral lipid mass transfer activity in plasma can be attributed to the same protein responsible for all radioisotopic neutral lipid transfer activity in plasma, LTP, since a single mAb, 3-9F, completely abolished both. Furthermore, the action of 14-8H on neutral lipid mass transfer activity in incubated plasma highlights certain features of the LTP neutral lipid transfer mechanism. Blockage of the approximate heteroexchange of neutral lipid between HDL and VLDL by 14-8H while allowing radioisotopic CE transfer can be interpreted as modification of the activity of LTP by 14-8H, effectively rendering it a CE exchange protein incapable of mass transfer. This is consistent with the hypothesis of a single neutral lipid binding site in LTP involved in neutral lipid transfer, which may be partially obstructed by 14-8H to prevent TG entry, while being inconsistent with the hypothesis for independent, separate sites for CE and TG binding and transfer, in which case 14-8H would be expected to allow CE mass transfer. Paradoxically, LTP may be able to facilitate unidirectional net neutral lipid transfer as well, as there seemed to be increase in LDL core lipid content after plasma incubation (Table 4.2), which could be inhibited by either mAb. Transfer of CE from HDL to TG/PL emulsion without equimolar back transfer of TG has been described (Hesler *et al.*, 1987), as well as unidirectional transfer between PL-cholesterol liposomes (Chajek *et al.*, 1980).

Changes in lipoprotein lipid levels following incubation of human plasma incubation without inactivation of LCAT were also examined. In this case, CE synthesis and net transfer of neutral lipids contribute to changes in lipoprotein CE levels. Compared to control samples kept at 4° C, in plasma incubated at 37° C for 24 h, VLDL TG decreased markedly, with accompanying increases in LDL and HDL TG levels (Table 4.4). Furthermore, CE levels increased in all the lipoprotein classes, including HDL, in which CE levels decreased when the incubation was performed with LCAT inhibited. Also, the change in LDL CE was not significant, whereas there was an unexpected increase when the incubation was performed with LCAT inhibited (Table 4.2). mAb 3-9F or 14-8H both effectively abolished changes in lipoprotein TG levels as well as increases in VLDL and LDL CE normally seen after incubation of untreated plasma. HDL CE levels were further elevated than during 37° C incubation alone of untreated plasma. Thus, with LCAT active, incubation of human plasma lead to increased levels of HDL CE, even with neutral lipid mass transfer occurring during incubation of the plasma. When neutral lipid mass transfer was abolished, which could be achieved by treatment with either 3-9F or 14-8H, HDL CE accumulated even further.

The amount of cholesterol esterification resulting from incubation of human plasma at 37° C for 24 h was not affected by abolition of neutral lipid mass transfer with mAb (Table 4.4), demonstrating that LTP mediated CE mass transfer out of HDL is not required for LCAT activity under near physiological conditions. In comparison, *in vitro* inhibition of LCAT activity due to product inhibition can be relieved by LTP activity (Chajek *et al.*, 1980) Furthermore, cholesterol esterification during incubation of plasma resulted in greater decreases in VLDL and LDL than HDL free cholesterol levels (Table 4.3). Thus, under our experimental conditions with LCAT active, HDL maintained its free cholesterol level while extracting free cholesterol from lower density lipoproteins, in absence of peripheral tissues and erythrocytes which would be sources *in vivo*. The free cholesterol level among the different lipoprotein classes appears to be in equilibrium in freshly isolated plasma since following incubation with LCAT inhibited, they did not change (Table 4.2).

4.3.6 Demonstration of Interaction Between mAbs and LTP

It was noted during their preparation that 2-8G and 3-9F gave only a weak signal during dot blotting to immobilized rabbit LTP whereas 14-8H reacted strongly. SDS-PAGE and immunoblotting with each mAb individually against purified rabbit and human LTP also revealed strong binding by 14-8H to denatured rabbit and human LTP, while binding of 2-8G and 3-9F were not detected (Fig. 4.6). Recognition of denatured rabbit and human LTP by TP2, a mAb raised against human LTP (Hesler *et al.*, 1988) could also be demonstrated (Fig 4.6, lanes 11, 12). Native-PAGE and immunoblotting was able to demonstrate direct interaction of all three mAbs to rabbit LTP, as well as the species-specificity of 2-8G for rabbit LTP by its lack of recognition of human LTP (Fig. 4.7). The interaction of 3-9F appeared weaker towards human LTP than rabbit LTP, but was clearly evident compared to the complete absence of interaction between 2-8G and human LTP. During our native-PAGE, both LTPs migrated slower than bovine serum albumin (66 kDa) but faster than transferrin (81 kDa), behavior consistent with LTP being monomeric under these conditions and as well, TP2 binding to rabbit and human LTP under these conditions was demonstrable (Fig. 4.7). Thus, 14-8H binds to an epitope stable to denaturation by SDS. The lack of binding of 2-8G and 3-9F to denatured antigen suggests that their interaction with LTP involves conformational epitopes maintained only by LTP in its native state.

4.4 Discussion

Three inhibitory mAbs raised against rabbit LTP: 2-8G, 3-9F and 14-8H were further characterized to ascertain their mechanisms for neutralization and dissociation of radioisotopic neutral lipid transfer by LTP. The effects of neutralizing mAbs 2-8G and 3-9F on LTP binding to lipid microemulsion particles suggest that they interfere with a structural determinant within LTP important for interacting with the lipoprotein surface during lipid transfer. The effects of dissociating mAb 14-8H on pyrene-neutral lipid transfer and mass transfer of neutral lipids within incubated human plasma suggest that it interferes with a protein determinant within LTP important for neutral lipid binding and transfer. Furthermore, they support the hypothesis of a heteroexchange mechanism for mass transfer of neutral lipids between lipoproteins, mediated through a common site (or overlapping sites) within LTP where CE and TG compete for mutually exclusive binding and transfer between lipoproteins. These novel mAbs should be useful for further characterizing the structural determinants of LTP activity.

4.4.1 Effects of mAbs on Lipid Particle Binding by LTP

LTP binds to lipoprotein mainly through electrostatic interactions with negative charges of PL headgroups on the lipoprotein surface (Pattnaik and Zilversmit, 1979). Modifications of the lipoprotein surface using various approaches showed that optimum transfer activity depends on a fine balance of ionic interactions (Nishida *et al.*, 1993). Moreover, lipoprotein binding is increased by binding of a mAb to the C-terminal region of LTP involved in neutral lipid binding and transfer, presumably through a conformational change in the putative lipoprotein binding domain (Swenson *et al.*, 1989). Deletion mutagenesis has also defined another region near the C-terminal of LTP which is required for lipoprotein binding and lipid transfer by LTP (Au-Young and Fielding, 1992). Furthermore, the N-terminal domain of BPI has been shown to interact with heparin, as well as being largely responsible for LPS binding through interactions with the negatively charged lipid A moiety (Little *et al.*, 1994), which may be analogous to PL binding by LTP.

mAbs 2-8G and 3-9F interfered with LTP binding to different degrees. Moreover, 2-8G was species-specific for rabbit LTP. This presumably arises through species differences in protein sequence between human and rabbit LTP, which is 20 amino acids longer mainly due to a 19 residue insertion within the C-terminal portion of rabbit LTP (Nagashima *et al.*, 1988). Otherwise, the proteins are highly similar, with 81% sequence identity, and with two-thirds of the substitutions being conservative. The sequences implicated to be involved in lipoprotein binding by human LTP from Wang *et al.* (1991) and Au-Young and Fielding (1992) results (see Introduction) are well conserved in rabbit LTP (Nagashima *et al.*, 1988). The epitope(s) recognized by mAbs which inhibit lipid particle binding by LTP are not stable to denaturation by SDS, suggesting that they depend on proper protein folding and tertiary structure of LTP.

Our results also suggest that 14-8H interferes with the neutral lipid binding and transfer site of LTP, and consistent with this, it did not influence microemulsion binding. LTP appears to bind tightly to microemulsions, perhaps greater than binding to lipoproteins, which is unaffected by apoA-I (Ohnishi *et al.*, 1994). While TP2 Fab enhanced lipoprotein binding by LTP, a similar effect of 14-8H on LTP microemulsion binding was not measurable because of already high binding in its absence.

4.4.2 Effects of mAbs on Lipid Transfer Activity of LTP

The C-terminal region of LTP is crucial for neutral lipid transfer activity by binding the neutral lipid substrate which will be eventually transferred (Swenson *et al.*, 1989). Inhibition of transfer activity by neutralizing mAb (Swenson *et al.*, 1989) or deletion mutagenesis of LTP (Wang *et al.*, 1992) suggest that CE and TG transfer are linked. Previous studies showing that CE and TG compete for transfer also suggest that CE and TG transfer activities are inter-related (Morton and Zilversmit, 1983). Moreover, the observation that net mass transfer between lipoprotein pairs not at equilibrium is approximately equimolar (Nichols and Smith, 1965; Whitlock *et al.*, 1989), suggests that a reciprocal heteroexchange mechanism is involved in neutral lipid net mass transfer.

Our results are consistent with a heteroexchange mechanism for neutral lipid net mass transfer by LTP and our 14-8H seems to behave similar to the TP2 Fab fragment, which dissociates radioisotopic neutral lipid transfer presumably by binding within the neutral lipid binding site of LTP, preventing entry of TG (Swenson *et al.*, 1988). The net result of 14-8H action would be to allow CE homoexchange between any lipoprotein pair, which can be shown by radioisotopic methods, while abolishing the net neutral lipid transfer between lipoprotein pairs differing in core neutral lipid ratio, which requires heteroexchange to occur (Fig. 4.8). Although effects of TP2 Fab fragment on neutral lipid net mass transfer have not been reported, it would be expected to neutralize CE and TG mass transfer, as whole TP2 does (Yen *et al.*, 1989). Others (Fukasawa *et al.*, 1992; Melchior *et al.*, 1995) also reported production of a dissociating mAb, similar to 14-8H in effects on radioisotopic neutral lipid transfer, although further information about effects on neutral lipid net mass transfer are lacking. Thus it seems relatively common to generate mAbs against the neutral lipid binding site of LTP which recognize epitopes stable to SDS denaturation. The C-terminal region of LTP (and related proteins) is highly hydrophobic, with sections of predicted alpha-helical structure possibly involved in lipid binding (reviewed in Tall, 1993)

On the other hand, modification of LTP sulfhydryl groups with pCMPS produced dissociation of radioisotopic neutral lipid transfer (see Introduction), suggesting that CE and TG transfer were independent. Effects on neutral lipid net mass transfer have been reported, with one report of total inhibition (Hopkins and Barter, 1980), consistent with a heteroexchange mechanism, while a later report stated that mass transfer was also independently affected (Hopkins and Barter, 1982). There is no Cys in the C-terminal region of human LTP containing the TP2 epitope and important in neutral lipid binding and transfer (Wang *et al.*, 1992), suggesting that other regions of LTP contribute to the formation of the neutral lipid binding site. Furthermore, other less hydrophobic sulfhydryl reagents such as DTNB do not inhibit or dissociate LTP activity. Chemical compounds resembling cholesterol have also been shown to selectively inhibit CE transfer rather than TG (see Introduction). However, their effects on neutral lipid mass transfer have not been determined, and their similarities to cholesterol suggests that they may merely be somehow competing with CE to limit its transfer. Perhaps they have higher affinity than CE for LTP, or alternatively, they may alter the lipoprotein surface properties so that less CE is present, while not in themselves being transfer substrates. Thus, the apparent dissociation of radioisotopic neutral lipid transfer by these compounds should not necessarily be taken as proof of independent neutral lipid transfer sites within LTP.

Table 4.1. Effects of mAbs on rate constants for the transfer of pyrene-lipids by rabbit and human LTP between lipid microemulsion particles.

Pure rabbit and human LTP (0.5 μg for pyrene-CE transfer and 1 μg for pyrene-TG transfer, respectively) were treated with each mAb (5 μg of IgG) before performing pyrene-lipid assay in the presence of apoA-I. The results are typical of 2 trials.

Lipid transfer in the presence of:	Rate constant $(k/L_1) \times 10^3$			
	Rabbit LTP		Human LTP	
	pyrene-CE	pyrene-TG	pyrene-CE	pyrene-TG
ApoA-I alone (no LTP)	1.5	3.0	1.5	3.0
LTP plus apoA-I	28.2	9.3	68.1	25.7
" " " and 2-8G	3.3	3.2	40.1	19.2
" " " and 3-9F	0.6	3.4	2.0	3.4
" " " and 14-8H	4.0	4.5	3.4	4.2

Table 4.2. Effects of mAbs on changes in lipoprotein lipid composition following incubation of human plasma with LCAT inhibited.

Plasma samples ($n = 6$) were pretreated with DTNB, followed by mAb treatment and incubation as indicated. Then analysis of lipoprotein lipid composition was performed as described in "Experimental Procedures." All values are means (SEM) in mg/dL and statistically significant differences between 37° C, without or with mAb and 4° C control values are shown.

Lipoprotein Lipid:	Incubation Conditions			
	4° C control	37° C, no mAb	37° C with 3-9F	37° C with 14-8H
VLDL				
Free cholesterol	7.1 (1.2)	7.9 (1.4)^a	8.7 (1.6)^a	8.3 (1.4)^a
CE	12.5 (5.3)	22.0 (11.4)^a	13.9 (6.5)	14.4 (6.0)^a
TG	58.2 (15.2)	37.3 (10.5)^b	60.2 (16.0)	55.4 (13.4)
LDL				
Free cholesterol	20.3 (0.9)	22.9 (1.4)^a	20.6 (1.3)	20.9 (1.6)
CE	75.3 (9.7)	82.6 (12.8)	81.3 (11.2)^a	80.0 (14.3)
TG	13.9 (2.4)	28.1 (6.4)^a	15.9 (1.8)	12.9 (3.7)
HDL				
Free cholesterol	19.2 (0.6)	19.0 (0.5)	19.7 (0.5)	19.1 (0.3)
CE	38.7 (5.9)	33.9 (4.2)^a	40.8 (4.9)	37.8 (5.1)
TG	26.9 (1.9)	36.7 (1.6)^c	27.1 (2.0)	31.0 (2.7)
Total				
Free cholesterol	46.6	49.9	48.9	48.3
CE	126.5	138.4	135.9	132.2
TG	99.0	102.1	103.2	99.2

$^a P < 0.05$
$^b P < 0.01$
$^c P < 0.001$

Table 4.3. Changes in lipoprotein neutral lipid levels on a molar basis in incubated human plasma with LCAT inactivated.

Data from Table 4.2 (37° C, no mAb minus 4° C control values) were converted to mM using molecular weights of 387 for cholesterol moiety and 850 for TG.

Lipoprotein:	Net change following incubation (mM)
VLDL	
CE	+ 0.245
TG	- 0.246
LDL	
CE	+ 0.189
TG	+ 0.167
HDL	
CE	- 0.124
TG	+ 0.115

Table 4.4. Effects of mAbs on changes in lipoprotein lipid composition following incubation of human plasma without LCAT inhibited.

Plasma samples (*n* = 6) were treated with mAb and incubation as indicated. Then analysis of lipoprotein lipid composition was performed as described in "Experimental Procedures." All values are means (SEM) in mg/dL and statistically significant differences between 37° C, without or with mAb and 4° C control values are shown.

Lipoprotein Lipid:	Incubation Conditions			
	4° C control	37° C, no mAb	37° C with 3-9F	37° C with 14-8H
VLDL				
Free cholesterol	7.0 (1.3)	3.1 (0.6)^{a}	4.8 (0.9)^{a}	4.4 (0.7)^{a}
CE	12.5 (2.2)	19.0 (3.5)^{a}	13.7 (2.0)	15.2 (2.2)^{b}
TG	58.2 (15.5)	29.7 (10.4)^{a}	61.2 (17.8)	53.1 (14.2)^{c}
LDL				
Free cholesterol	20.3 (1.6)	7.8 (2.2)^{a}	5.7 (1.3)	5.7 (1.2)^{b}
CE	75.3 (4.5)	83.5 (5.8)	76.0 (4.9)	77.3 (5.2)
TG	13.9 (3.1)	25.7 (6.6)^{c}	13.1 (2.9)	16.7 (4.3)
HDL				
Free cholesterol	19.2 (1.0)	14.8 (0.6)	15.0 (0.5)	15.0 (0.3)^{a}
CE	38.7 (2.3)	46.0 (3.3)^{c}	55.9 (4.3)^{a}	52.1 (3.8)^{a}
TG	26.9 (2.0)	36.4 (2.5)^{b}	26.3 (1.8)	28.3 (1.3)
Total				
Free cholesterol	46.6	25.7	25.4	25.1
CE	126.5	148.4	145.6	144.6
TG	99.0	91.8	100.7	98.2

$^{a}P < 0.05$

$^{b}P < 0.01$

$^{c}P < 0.001$

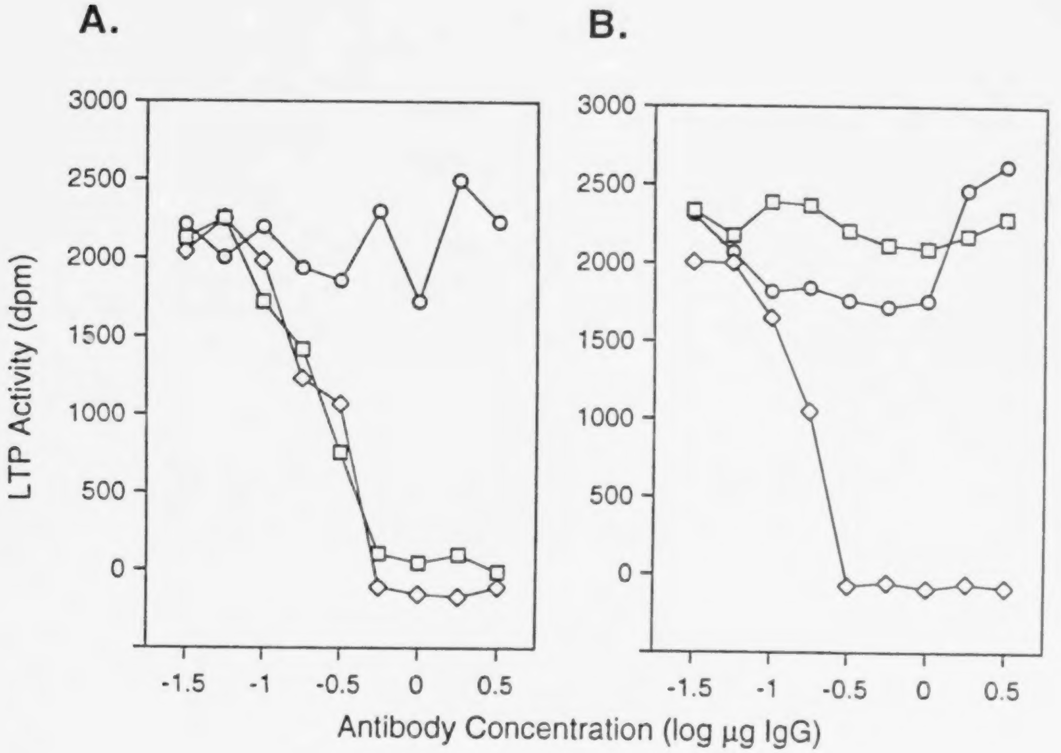


Figure 4.1. Immunotitration of CE transfer mediated by rabbit (panel A) and human (panel B) LTP. Rabbit (25 µL) or human (50 µL) LPDP were treated with indicated amounts of IgG fraction containing mAb 2-8G (squares), 3-9F (diamonds), or 14-8H (circles) before performing a radioisotopic CE transfer assay on the mixtures. In the absence of mAb, control rabbit LTP CE transfer activity was 2,403 dpm and control human LTP CE transfer activity was 2,352 dpm. Shown are typical results of 3 trials.

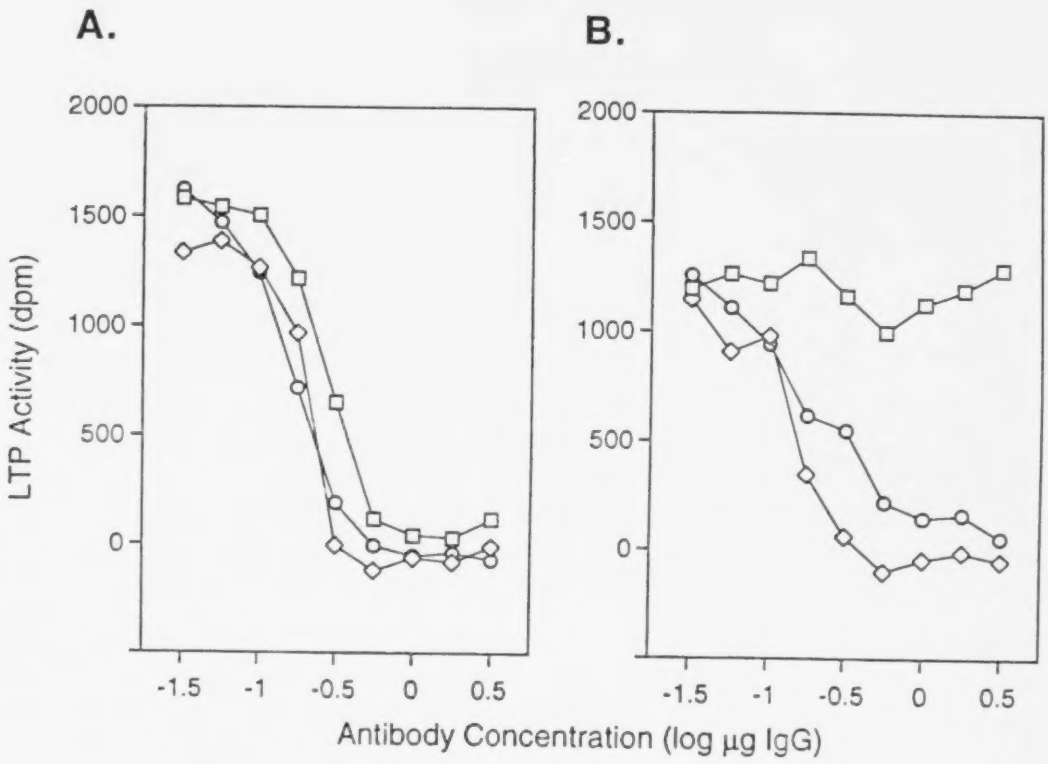


Figure 4.2. Immunotitration of TG transfer mediated by rabbit (panel A) and human (panel B) LTP. Treatments of rabbit and human LPDP with various mAbs, and meanings of the symbols, are as described in Fig. 4.1 before a radioisotopic TG transfer assay was performed on the mixtures. In the absence of mAb, control rabbit LTP TG transfer activity was 1,736 dpm and control human LTP TG transfer activity was 1,223 dpm. Shown are typical results of 3 trials.

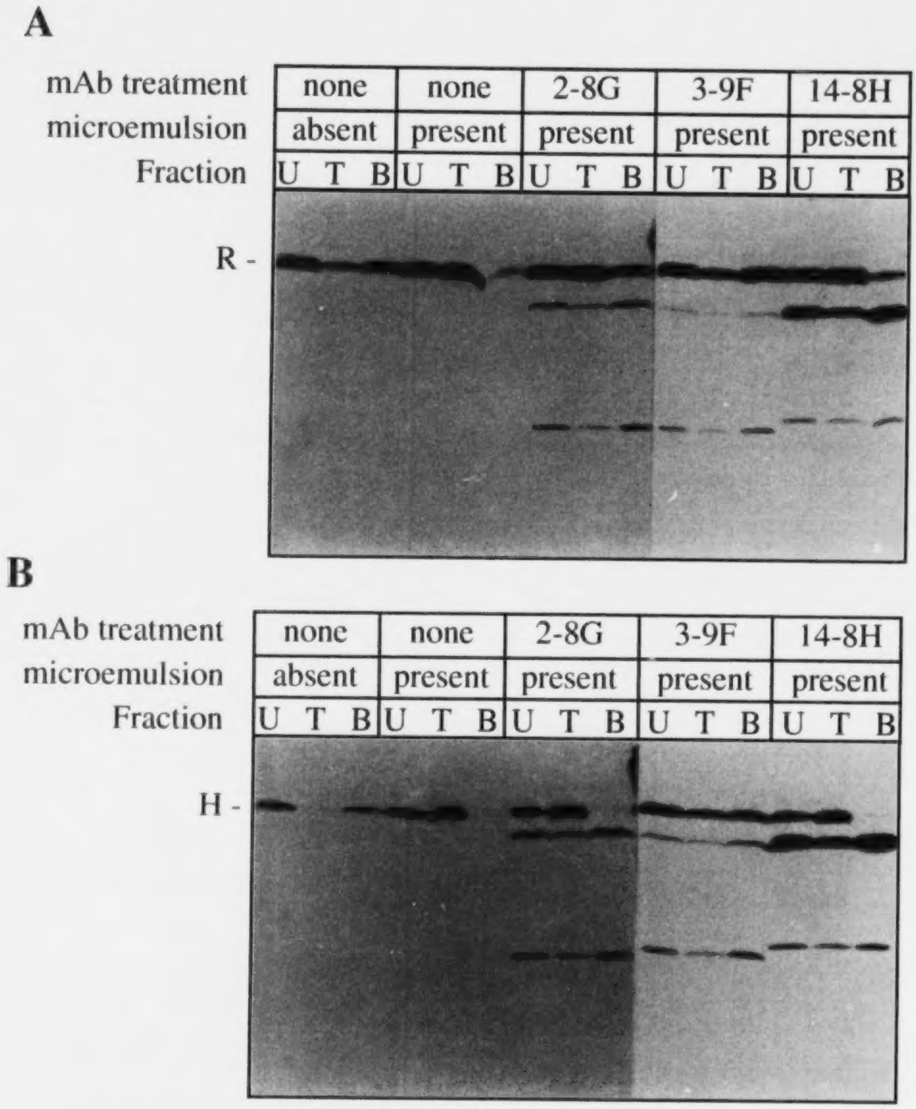

A
mAb treatment
none
none
2-8G
3-9F
14-8H
microemulsion
absent
present
present
present
present
Fraction
U T B
U T B
U T B
U T B
U T B
R -
B
mAb treatment
none
none
2-8G
3-9F
14-8H
microemulsion
absent
present
present
present
present
Fraction
U T B
U T B
U T B
U T B
U T B
H -

Figure 4.3. Effects of mAbs on LTP binding to lipid microemulsion particles. Following treatment of rabbit (panel A) and human (panel B) LTP with the indicated mAb, microemulsions were added to the indicated samples. After incubation, the unspun (U) fraction was sampled before ultracentrifugation followed by separation of tube contents into top (T) and bottom (B) half fractions. Shown are fluorographs after SDS-PAGE and immunoblotting, using 14-8H as the primary antibody, of each fraction. R and H on the sides denote the positions of rabbit and human LTP, respectively. The lower M_r bands are mAb heavy and light chains, which were also recognized by the secondary antibody. The results are typical of 2 trials.

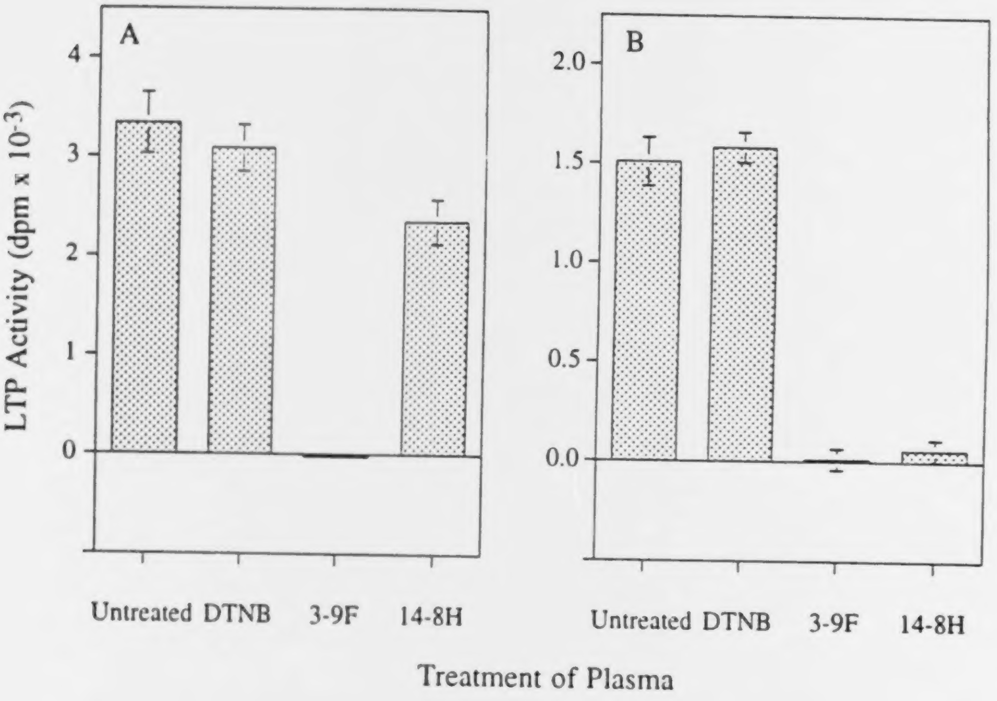


Figure 4.4. Effects of DTNB or mAb treatment on radioisotopic neutral lipid transfer activity stimulated by human plasma. Following treatment of human plasma with DTNB or mAbs as described in "Experimental Procedures," samples (n = 3) were subjected to radioisotopic CE (panel A) and TG (panel B) assays. Error bars indicate SEM of the data.

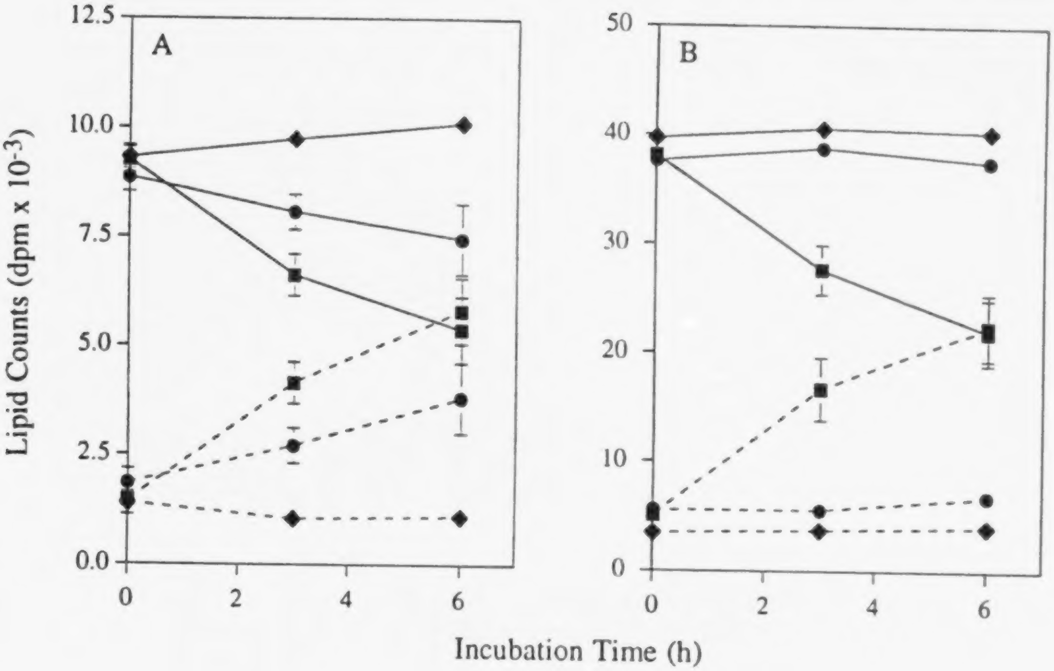


Figure 4.5. Effects of mAbs on radioisotopic CE (panel A) and TG (panel B) transfer between endogenous lipoproteins within incubated human plasma. Human plasma samples (n = 3), untreated (squares) or pretreated with mAb 3-9F (diamonds) or 14-8H (circles), were spiked with HDL containing radiolabeled CE or TG followed by incubation and determination of counts in HDL (solid lines) and lower density lipoproteins (dashed lines) as described in "Experimental Procedures." Error bars indicate SEM of the data.

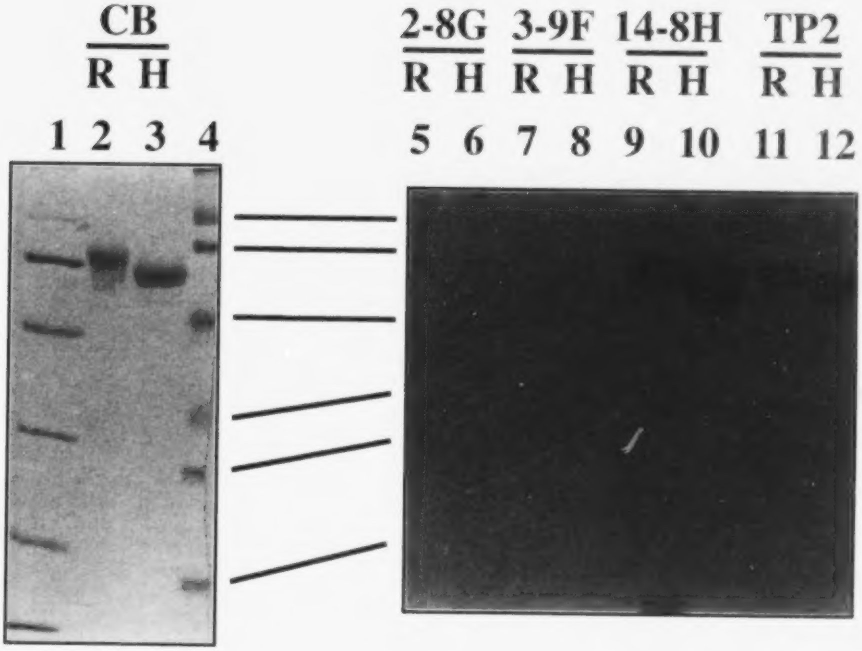


Figure 4.6. SDS-PAGE and immunoblotting of LTP. LTP (1 μg) was subjected to SDS-PAGE, followed by Coomassie Blue (CB) staining of the gel (lanes 1-4) or immunoblotting of LTP (lanes 5-12) using the indicated mAb (30 μg IgG/mL) as the primary antibody. R and H denote the source species of LTP, rabbit and human, respectively. Lanes 1 and 4 contain Bio-Rad SDS -PAGE Molecular Weight Standards (Low Range) and Prestained SDS-PAGE Standards (Low Range), respectively, and the bars mark the respective positions of the prestained standards on the gel and the nitrocellulose membrane. The results are typical of 3 trials.

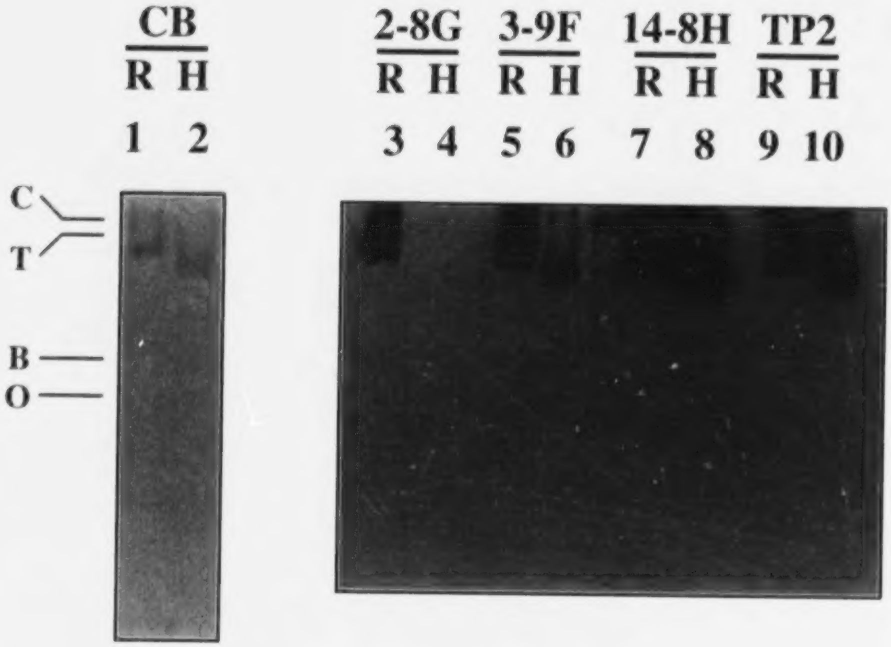


Figure 4.7. Native-PAGE and immunoblotting of LTP. LTP (1 μg) was subjected to native-PAGE followed by Coomassie Blue (CB) staining of the gel (lanes 1, 2) or immunoblotting of LTP (lanes 3-10) as described in Fig. 4.6. Again, R and H denote rabbit and human LTP, respectively. The migration positions of catalase (C, 220 kDa), transferrin (T, 81 kDa), bovine serum albumin (B, 66 kDa) and ovalbumin (O, 43 kDa) in the same gel are as indicated. The results are typical of 3 trials.

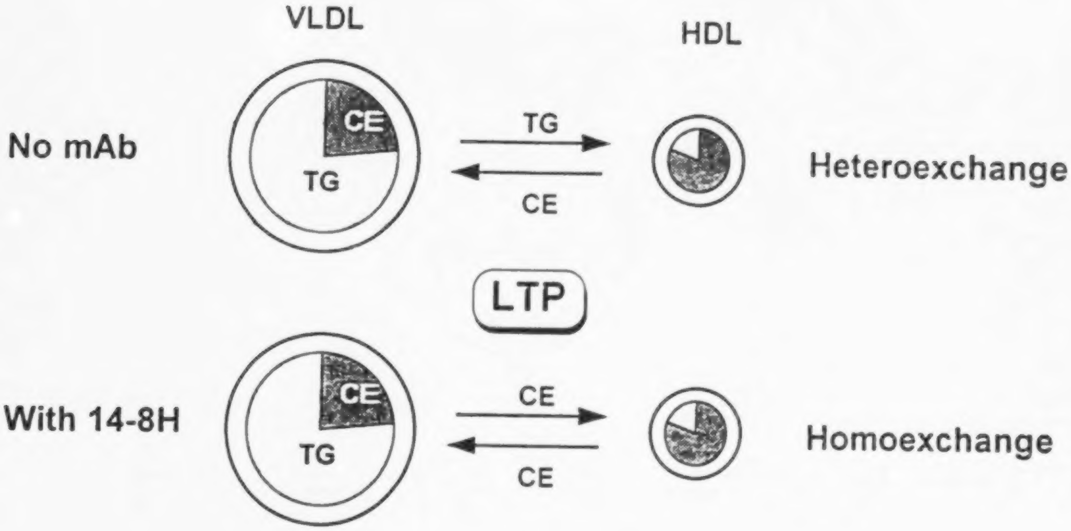


Figure 4.8. How mAb 14-8H allows radioisotopic but not net mass transfer of CE. Under normal circumstances (no mAb), LTP mediates the net transfer of CE from HDL to VLDL with transfer of TG in the opposite direction. According to the single site model for neutral lipid transfer by LTP, this occurs through a heteroexchange mechanism, and depends on the difference in core neutral lipid ratios of the donor and acceptor lipoproteins. Treatment (with mAb 14-8H) appears to hinder entry of TG into the single site for neutral lipid binding and transfer, rendering LTP capable only of CE homoexchange, which can be shown radioisotopically, but not net mass transfer of CE (or TG), as shown by mass measurements, irrespective of the core neutral lipid ratios of the donor and acceptor lipoproteins.

4.5 References

Au-Young, J., and Fielding, C.J. (1992) Synthesis and secretion of wild-type and mutant human plasma cholesteryl ester transfer protein in baculovirus-transfected insect cells: the carboxyl-terminal region is required for both lipoprotein binding and catalysis of transfer. *Proc. Natl. Acad. Sci. USA* 89, 4094-4098

Bisgaier, C.L., Essenburg, A.D., Minton, L.L., Homan, R., Blankley, C.B. *et al.*, (1994) Cholesteryl ester transfer protein inhibition by PD 140195. *Lipids* 29, 811-818

Busch, S.J., and Harmony, J.A.K. (1990) Cholesteryl ester analogs inhibit cholesteryl ester but not triglyceride transfer catalyzed by the plasma cholesteryl ester-triglyceride transfer protein. *Lipids* 25, 216-220

Chajek, T., Aron, L., and Fielding, C.J. (1980) Interaction of lecithin:cholesterol acyltransferase and cholesteryl ester transfer protein in the transport of cholesteryl ester into sphingomyelin liposomes. *Biochemistry* 19, 3673-3677

Drayna, D., Jarnagin, A.S., McLean, J., Henzel, W., Kohr, W. *et al.* (1987) Cloning and sequencing of human cholesteryl ester transfer protein cDNA. *Nature* 327, 632-634

Epps, D.E., Greenlee, K.A., Harris, J.S., Thomas, E.W., Castle, C.K. *et al.* (1995) Kinetics and inhibition of lipid exchange catalyzed by plasma cholesteryl ester transfer prtoein (lipid transfer protein). *Biochemistry* 34, 12560-12569

Fukasawa, M., Arai, H., and Inoue, K. (1992) Establishment of anti-human cholesteryl ester transfer protein monoclonal antibodies and radioimmunoassaying of the level of cholesteryl ester transfer protein in human plasma. *J. Biochem.* 111, 696-698

Gallay, P., Heumann, D., Le Roy, D., Barras, C., and Glausner, M.P. (1993) Lipopolysaccharide-binding protein as a major plasma protein for endotoxemic shock. *Proc. Natl. Acad. Sci USA* 90, 9935-9938

Gray, P.W., Flaggs, G., Leong, S.R., Gumina, R.J., Weiss, J. *et al.* (1989) Cloning and cDNA of a human neutrophil bactericidal protein. Structural and functional correlations. *J. Biol. Chem.* 264, 9505-9509

Harlow, E., and Lane, D. (1988) Storing and purifying antibodies. In *Antibodies: A lab manual* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 283-318

Hesler, C.B., Swenson, T.L., and Tall, A.R. (1987) Purification and characterization of a human plasma cholesteryl ester transfer protein. *J. Biol. Chem.*. 262, 2275-2282

Hesler, C.B., Tall, A.R., Swenson, T.L., Weech, P.K., Marcel, Y.L. *et al.* (1988) Monoclonal antibodies to the M_r 74,000 cholesteryl ester transfer protein neutralize all of the cholesteryl ester and triglyceride transfer activities in human plasma. *J. Biol. Chem.* 263, 5020-5023

Hopkins, G.J., and Barter, P.J. (1980) Transfers of esterified cholesterol and triglyceride between high density and very low density lipoproteins: *in vitro* studies of rabbits and humans. *Metabolism* 29, 546-550

Hopkins, G.J., and Barter, P.J. (1982) Dissociation of the in vitro transfers of esterified cholesterol and triglyceride between human lipoproteins. *Metabolism* 31, 78-81

Jin, H., Yang, R., Marsters, S., Ashkenazi, A., Bunting, S. *et al.* (1995) Protection against endotoxic shock by bactericidal/permeability-increasing protein in rats. *J. Clin. Invest.* 95, 1947-1952

Ko, K.W.S., Oikawa, K., Ohnishi, T., Kay, C.M., and Yokoyama, S. (1993) Purification, characterization, and conformational analysis of rabbit plasma lipid transfer protein. *Biochemistry* 32, 6729-6736

Little, R.G., Kelner, D.N., Lim, E., Burke, D.J., and Conlon, P.J. (1994) Functional domains of recombinant bactericidal/permeability increasing protein (rBPI23). *J. Biol. Chem.* 269, 1865-1872

Lowry, O.L., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) Protein measurement with the folin phenol reagent. *J. Biol. Chem.* 193, 265-275

Melchior, G.W., Greelee, K.A., Castle, C.K., Prough, M.J., Milne, R.W. *et al.* (1995) Evidence that cynomolgus monkey cholesteryl ester transfer protein has two neutral lipid binding sites. *J. Biol. Chem.* 270, 21068-21074

Morton, R.E., and Zilversmit, D.B. (1982) Purification and characterization of lipid transfer protein(s) from human lipoprotein-deficient plasma. *J. Lipid Res.* 23, 1058-1067

Morton, R.E., and Zilversmit, D.B. (1983) Inter-relationship of lipids transferred by the lipid-transfer protein isolated from human lipoprotein-deficient plasma. *J. Biol. Chem.* 258, 11751-11757

Nagashima, M., McLean, J.W., and Lawn, R.M. (1988) Cloning and mRNA tissue distribution of rabbit cholesteryl ester transfer protein. *J. Lipid Res.* 29, 1643-1649

Nichols, A.V., and Smith, L. (1965) Effect of very low-density lipoproteins on lipid transfer in incubated serum. *J. Lipid Res.* 6, 206-210

Nishida, H.I., Arai, H., and Nishida, T. (1993) Cholesteryl ester transfer mediated by lipid transfer protein as influences by changes in the charge characteristics of plasma lipoproteins. *J. Biol. Chem.* 268, 16352-16360

Ohnishi, T., and Yokoyama, S. (1993) Activation of human lipid transfer protein by apolipoproteins. *Biochemistry* 32, 5029-5035

Ohnishi, T., Yokoyama, S., and Yamamoto, A. (1990) Rapid purification of human plasma lipid transfer protein. *J. Lipid Res.* 31, 397-406

Ohnishi, T., Hicks, L.D., Oikawa, K., Kay. C.M., and Yokoyama, S. (1994) Properties of human plasma lipid transfer protein in aqueous solution and at interfaces. *Biochemistry* 33, 6093-6099

Ooi, C.E., Weiss, J., Elsbach, P., Frangione, B., and Mannion, B. (1987) A 25-kDa NH_2-terminal fragment carries all the antibacterial activities of the human neutrophil 60-kDa bactericidal/permeability-increasing protein. *J. Biol. Chem.* 262, 14891-14894

Ooi, C.E., Weiss, J., Doerfler, M.E., and Elsbach, P. (1991) Endotoxin-neutralizing properties of a 25 kD N-terminal fragment and a newly isolated 30 kD C-terminal fragment of the 55-60 kD bactericidal/permeability-increasing protein of human neutrophils. *J. Exp. Med.* 174, 649-655

Pattnaik, N.M., and Zilversmit, D.B. (1979) Interaction of cholesteryl ester exchange protein with human plasma lipoproteins and phospholipid vesicles. *J. Biol. Chem.* 254, 2782-2786

Swenson, T.L., Brocia, R.W., and Tall, A.R. (1988) Plasma cholesteryl ester transfer protein has binding sites for neutral lipids and phospholipids. *J. Biol. Chem.* 263, 5150-5157

Swenson, T.L., Hesler, C.B., Brown, M.L., Quinet, E., Trotta, P.P. *et al.* (1989) Mechanism of cholesteryl ester transfer protein inhibition by a neutralizing monoclonal antibody and mapping of the monoclonal antibody epitope. *J. Biol. Chem.* 264, 14318-14326

Tall, A.R. (1993) Plasma cholesteryl ester transfer protein. *J. Lipid Res.* 34, 1255-1274

Tall, A.R., Granot, E., Brocia, R., Tabas, I., Hesler, C. *et al.* (1987) Accelerated transfer of cholesteryl esters in dyslipidemic plasma. Role of cholesteryl ester transfer protein. *J. Clin. Invest.* 79, 1217-1225

Tobias, P.S., Soldau, K., and Ulevitch, R.J. (1986) Isolation of a lipopolysaccharide-binding reactant from rabbit serum. *J. Exp. Med.* 164, 777-793

Tobias, P.S., Soldau, K., and Ulevitch, R.J. (1989) Identification of a lipid A binding site in acute phase reactant lipopolysaccharide binding protein. *J. Biol. Chem.* 264, 10867-10871

Wang, S., Deng, L., Brown, M.L., Agellon, L.B., and Tall, A.R. (1991) Structure-function studies of human cholesteryl ester transfer protein by linker insertion scanning mutagenesis. *Biochemistry* 30, 3484-3490

Wang, S., Deng, L., Milne, R.W., and Tall, A.R. (1992) Identification of a sequence within the C-terminal 26 amino acids of cholesteryl ester transfer protein responsible for binding a neutralizing monoclonal antibody and necessary for neutral lipid transfer activity. *J. Biol. Chem.* 267, 17487-17490

Whitlock, M.E., Swenson, T.L., Ramakrishnan, R., Leonard, M.T., Marcel, Y.L. *et al.* (1989) Monoclonal antibody inhibition of cholesteryl ester transfer protein activity in the rabbit. Effects on lipoprotein composition and high density lipoprotein cholesteryl ester metabolism. *J. Clin. Invest.* 84, 129-137

Wright, S.D., Ramos, R.A., Tobias, P.S., Ulevitch, P.S., and Matheson, J.C. (1990) CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. *Science* 249, 1431-1433

Yen, F.T., Deckelbaum, R.J., Mann, C.J., Marcel, Y.L., Milne, R.W. *et al.* (1989) Inhibition of cholesteryl ester transfer protein activity by monoclonal antibody. Effects on cholesteryl ester formation and neutral lipid mass transfer in human plasma. *J. Clin. Invest.* 83, 2018-2024

CHAPTER 5

Discussion

5.1 General Discussion

Much attention has been drawn recently to proteins originally identified as having *in vitro* lipid transfer activity. Yeast phosphatidylinositol-transfer protein/SEC14p was found to be essential for viability and Golgi function (see introduction of Skinner *et al.*, 1995). Microsomal TG transfer protein is required for assembly of TG-rich lipoproteins, with its genetic defect being the cause of abetalipoproteinemia (reviewed in Gordon *et al.*, 1995). Before that, cytosolic PL transfer proteins have been described in plants (reviewed in Arondel and Kader, 1990) and mammals (reviewed in Wirtz and Gadella Jr, 1990), where they have been postulated to function in membrane biogenesis and interorganelle lipid transport (reviewed in Voelker, 1990), respectively.

Mammalian plasma LTP also belongs to the above group of proteins, sharing the ability to mediate transfer of hydrophobic lipids across an aqueous barrier between donor and lipid pools. The intrinsic ability of LTP to facilitate the exchange and net mass transfer of neutral lipids *in vitro* translates to physiological roles in modulating lipoprotein profile and metabolism and in RCT, thus presenting important implications towards atherosclerosis and CHD. Therefore, further investigations into the precise role and regulation of LTP activity should be valuable in combatting this major medical problem of industrialized nations, in addition to improving our basic understanding about mammalian plasma lipid transport and lipid-protein interactions. Interestingly, insects employ an analogous lipid transfer protein complex for modification of lipophorins within the hemolymph of their circulatory lipid transport system (reviewed in Ryan, 1994).

This thesis further investigated one of the many possible aspects in the regulation of LTP activity, which was the influence of apolipoproteins (Chapter 2). With a novel assay system employing pyrene-neutral lipids incorporated within lipid microemulsion particles to trace lipid transfer, regulation of LTP activity by the presence of exchangeable, amphiphilic alpha-helical apolipoproteins was clearly demonstrated. Activation of LTP seemed to be a general, dose-dependent effect of all the apolipoproteins tested, suggesting that physiologically, fluctuations in levels of certain apolipoproteins such as apoA-IV, which rises after fat feeding (Weinberg *et al.*, 1990), and apoC-III may be involved in fine-tuning of LTP activity. Understanding the precise mechanism for this activating effect of apolipoproteins will require further investigation.

There is little protein sequence homology between LTP and the other proteins with lipid transfer activity mentioned above. Instead, LTP belongs to a family of LPS binding proteins, with other members including BPI, LBP, and PLTP. LPS binding seems to be mediated by sequences within the N-terminal region of the protein. These last proteins and LTP also share the common feature of binding to lipoproteins or membranes, which seems to be a conserved function closely linked to the otherwise diverse biological actions of the various members of this family in bacterial killing, cell signaling, and lipid transfer. Lipoprotein or membrane binding is postulated to occur mainly through electrostatic interactions between PL and cationic residues within LTP, with some recent evidence in support (Jiang *et al.*, 1995). Protein structural molecular determinants of distinctive functions, such as the neutral lipid transfer activity of LTP, seem to localize to the C-terminal region of the protein, whereas details about PL transfer activity are less well characterized. On the other hand, the antibacterial activity of BPI is associated with the N-terminal portion of the protein (Ooi *et al.*, 1987) and little seems known about the molecular determinants involved in interaction of LBP with CD14.

LTP was isolated from rabbit plasma and shown to be biochemically very similar to the human form (Chapter 3). Thus, rabbit is a useful alternative source of LTP for mechanistic studies, as well offering a good animal model for investigating the *in vivo* roles of LTP. Protein structural analysis of rabbit LTP performed in conjunction with microemulsion binding studies revealed a high beta secondary structure content as well as affirming the structural stability of LTP upon lipid particle surface binding, in accordance with the definition of a Group 3 apolipoprotein (exchangeable, non alpha-helical forming upon lipid surface binding, able to bind and transfer lipids).

The details of the mechanism of the neutral lipid transfer function of LTP are being gradually unraveled. While biochemical approaches using kinetic studies, protein modification reagents, and chemical inhibitors have spurred controversy as to whether CE and TG transfer activities are interrelated or mediated by independent sites within LTP, the results from studies employing recombinant LTP and/or mAbs (Roy *et al.*, 1996) continue to support the existence of a single site involved in neutral lipid binding and transfer, along with the importance of a separate site involved in lipoprotein binding (Jiang *et al.*, 1995).

We were also successful in raising mAbs which affected LTP function (Chapter 4). Characterization of their mechanisms for inhibition of lipid transfer activity indicated that the mAbs obtained were novel. mAb 14-8H appears to be directed against the LTP molecular determinant for neutral lipid binding and transfer, binding in such a way as to dissociate radioisotopic neutral lipid transfer as does pCMPS or TP2 Fab treatment. Its total inhibition of changes in lipoprotein neutral lipid composition in incubated human plasma provided further evidence of a heteroexchange mechanism for net neutral lipid mass transfer by LTP. The effects of mAbs 2-8G and 3-9F on LTP binding to lipid microemulsion particles suggest that they target the molecular determinant of LTP involved in interaction with the lipoprotein surface, possibly the first case of such mAbs being produced. These mAbs and the microemulsion system should provide useful tools for further characterization of the neutral lipid binding site and the lipoprotein binding site of LTP.

An overall model summarizing the mechanism of LTP in transferring neutral lipids between lipoproteins is depicted, based on infomation from our current studies and existing background on LTP (Fig. 5.1). The active conformation of LTP is a globular structure with at least three functional regions formed and maintained by correct protein folding. First, there is a single neutral lipid binding and transfer site able to bind TG and CE, formed by the C-terminal region of LTP containing the TP2 epitope (Swenson *et al.*, 1989), as well as other regions distant from the C-terminal region in LTP primary sequence (Roy *et al.*, 1996). Secondly, nearby is a lipoprotein binding site which may be comprised of a Phe-Leu-Leu-Leu consensus sequence (Au-young and Fielding, 1992) as well as positively charged residues within LTP (Morton, 1985; Jiang *et al.*, 1995). Thirdly, there is a separate site for PL binding and transfer (Swenson *et al.*, 1988; Wang et al., 1992). mAbs 2-8G and 3-9F seem to affect functioning of the lipoprotein binding site, resulting in total inhibition of LTP activity. mAb 14-8H acts somehow, perhaps binding outside, or partially within the neutral lipid binding site to block entry of TG but not CE into the site, rendering LTP capable only of CE homoexchange. Increasing the bulk of CE by joining a pyrene moiety to it makes it susceptible to 14-8H inhibition of binding and transfer by LTP. These events occur on the lipoprotein surface, where neutral lipids must traverse the PL monolayer to gain access to LTP. The mechanism for stimulation of LTP neutral lipid transfer activity by apolipoproteins remains unclear, and may involve effects of the apolipoprotein directly with LTP, as well as effects on lipoprotein structural properties.

The results described within this thesis mainly delineated two separate functions of LTP crucial to its lipid transfer activity: lipoprotein binding and neutral lipid binding. Further details about how apolipoproteins activate LTP or subsequent steps of lipid transfer, such as LTP acting as a soluble lipid carrier or forming a ternary complex between donor and acceptor lipoproteins to complete the transfer process, will require additional work. Our findings also emphasize the importance of employing neutral lipid mass transfer assays and other varied, independent tests in order to understand the question of whether single or independent sites are involved in neutral lipid transfer by LTP, rather than basing a hypothesis solely on one type of study, in this case the radioisotopic neutral lipid transfer assay.

5.2 Future Directions

The lipid microemulsion particle system allows one to study LTP activity under very controlled conditions of lipid and protein composition within the lipid transfer assay. This system should be further utilized to determine the nature of activation of LTP activity by apolipoproteins; as well it would be interesting to test the effects of LTP inhibitors and apoC-I fragment within this system. Furthermore, regulation of LTP by lipid factors such as lipoprotein cholesterol, free fatty acid, or oxidized lipid content could be analyzed using lipid microemulsion particles as a comparative system.

Strong binding of LTP to lipid microemulsion particles was demonstrated under conditions which permit LTP activity in terms of pyrene-neutral lipid transfer. A question is whether binding of LTP to lipid microemulsion particles is analogous to LTP binding to natural lipoproteins.

One way to examine whether these processes are related might be to label natural lipoproteins with pyrene-neutral lipids and then evaluate LTP mediated lipid transfer between nearly homogeneous donor-acceptor lipoprotein pairs, which is difficult to do with radioisotopic transfer assays. Binding of LTP to these lipoproteins should be measured to see if it occurs to the same extent as binding to microemulsions. Furthermore, the role of lipoprotein binding in transfer activity can also be evaluated under these circumstances to possibly gain insight on the basic mechanism for lipid transfer by LTP, in other words whether a carrier or ternary complex formation process is employed. Answering this question, however, will require innovative approaches.

The ability to generate distinct mAbs apparently targeted against different structural determinants of LTP crucial to lipid transfer activity, lipoprotein binding and neutral lipid binding, suggest many possible avenues for future work. Mapping the epitopes of these mAbs is an obvious goal and competitive binding assays using existing mAbs against LTP whose epitopes have been well characterized (Roy *et al.*, 1996) offers an approach. The ability of mAbs to block LTP activity suggest that rational drug design to inhibit LTP may be feasible, and our mAbs may provide key tools for developing such drugs through generation and use of anti-idiotypic mAbs. The suggestion that pyrene-neutral lipids are prevented from entering the neutral lipid binding site of LTP by one of our mAbs due to the size of the pyrene moiety can be tested by developing flourescent lipid transfer assays using less bulkier probes, for instance parinaric acid linked to cholesterol, which should behave more like radioisotopic CE in transfer assays.

Three dimensional structural determination of LTP should be pursued, for instance using X-ray crystallography. This requires large quantities of LTP, and isolation from rabbit plasma provides one possible source. In addition to workers in the LTP field, this information would be valuable for researchers of other proteins belonging to the same family of LPS-binding proteins, LBP and BPI. BPI and perhaps LTP may have therapeutic value in curtailing endotoxic shock through their LPS binding activity (Jin *et al.*, 1995), and further insight into this interaction would be afforded by detailed protein structural information on any member of this family. In addition, such information would be monumental for understanding how LTP interacts with lipoproteins and transfers neutral lipids.

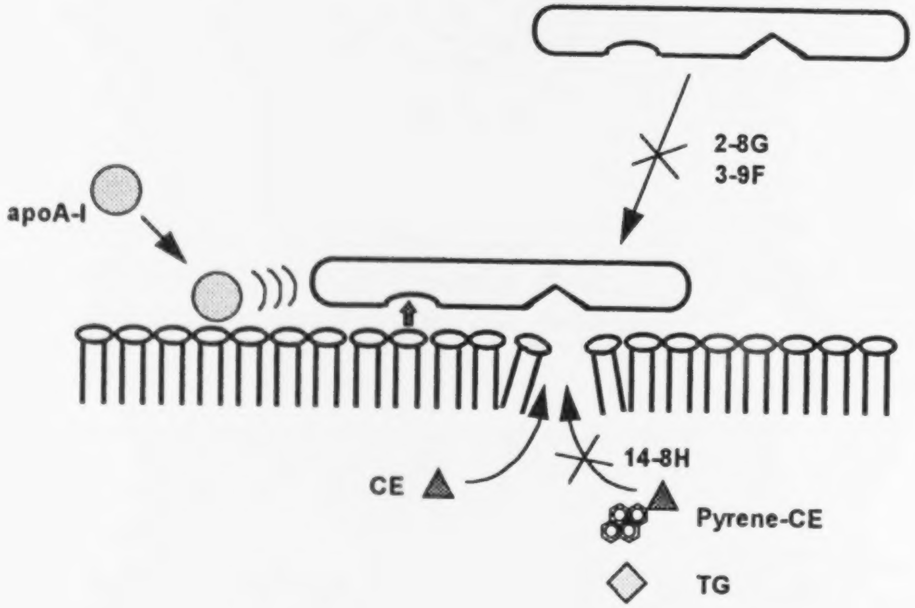

2-8G
3-9F
apoA-I
14-8H
CE
Pyrene-CE
TG

Figure 5.1. Overall model summarizing the mechanism and regulation of neutral lipid transfer by LTP. See text for description

5.3 References

Arondel, V., and Kader, J.C. (1990) Lipid transfer in plants. *Experientia* 46, 579-585

Au-Young, J., and Fielding, C.J. (1992) Synthesis and secretion of wild-type and mutant human plasma cholesteryl ester transfer protein in baculovirus-transfected insect cells: the carboxyl-terminal region is required for both lipoprotein binding and catalysis of transfer. *Proc. Natl. Acad. Sci. USA* 89, 4094-4098

Gordon, D.A., Wetterau, J.R., and Gregg, R.E. (1995) Microsomal triglyceride transfer protein: a protein complex required for the assembly of lipoprotein particles. *Trends Cell Biol.* 5, 317-321

Jiang, X.-.c., Bruce, C., Cocke, T., Wang, S., Boguski, M. *et al.* (1995) Point mutagenesis of positively charged amino acids of cholesteryl ester transfer protein: conserved residues within the lipid transfer/lipopolysaccharide binding protyein family essential for function. *Biochemistry* 34, 7258-7263

Jin, H., Yang, R., Marsters, S., Ashkenazi, A., Bunting, S. *et al.* (1995) Protection against endotoxic shock by bactericidal/permeability-increasing protein in rats. *J. Clin. Invest.* 95, 1947-1952

Ooi, C.E., Weiss, J., Elsbach, P., Frangione, B., and Mannion, B. (1987) A 25-kDa NH_2-terminal fragment carries all the antibacterial activities of the human neutrophil 60-kDa bactericidal/permeability-increasing protein. *J. Biol. Chem.* 262, 14891-14894

Roy, P., MacKenzie, R., Hirama, T., Jiang, X.-C., Kussie, P. *et al.* (1996) Structure-function relationships of human cholesteryl ester transfer protein: analysis using monoclonal antibodies. *J. Lipid Res.* 37, 22-34

Ryan, R.O. (1994) The structures of insect lipoproteins. *Curr. Opin. Struct. Biol.* 4, 499-506

Skinner, H.B., McGee, T.P., McMaster, C.R., Fry, M.R., Bell, R.M. *et al.* (1995) The *Saccharomyces cerevisiae* phosphatidylinositol-transfer protein effects a ligand-dependent inhibition of choline-phosphate cytidylyltransferase activity. *Proc. Natl. Acad. Sci. USA* 92, 112-116

Swenson, T.L., Brocia, R.W., and Tall, A.R. (1988) Plasma cholesteryl ester transfer protein has binding sites for neutral lipids and phospholipids. *J. Biol. Chem.* 263, 5150-5157

Swenson, T.L., Hesler, C.B., Brown, M.L., Quinet, E., Trotta, P.P. *et al.* (1989) Mechanism of cholesteryl ester transfer protein inhibition by a neutralizing monoclonal antibody and mapping of the monoclonal antibody epitope. *J. Biol. Chem.* 264, 14318-14326

Voelker, D.R. (1990) Lipid transport pathways in mammalian cells. *Experientia* 46, 569-578

Wang, S., Deng, L., Milne, R.W., and Tall, A.R. (1992) Identification of a sequence within the C-terminal 26 amino acids of cholesteryl ester transfer protein responsible for binding a neutralizing monoclonal antibody and necessary for neutral lipid transfer activity. *J. Biol. Chem.* 267, 17487-17490

Weinberg, R.B., Dantzker, C., Patton, C.S. (1990) Sensitivity of serum apolipoprotein A-IV to changes in dietary fat content. Gastroenterology 98, 17-24

Wirtz, K.W.A., and Gadella Jr, T.W.J. (1990) Properties and modes of action of specific and non-specific phospholipid transfer proteins. *Experientia* 46, 592-598

APPENDIX

Derivation of Equation to Calculate the Rate Constant k/L_1

Calibration of E_{470}/E_{395} or f of the homogeneous particle can be expressed as $f = a(c/L) + b$ (Fig. 2.3), where c is the concentration of pyrene-CE and L is concentration of total core lipid (TG + CE). When the heights of the fluorescence emission peaks of the monomer in the donor and acceptor particles are m_1 and m_2 and those of the excimer are e_1 and e_2 at incubation time t, the value of f measured for the incubation mixture is $f = (e_1 + e_2)/(m_1 + m_2)$. Because fluorescence intensity is proportional to concentration of pyrene compound within a particular solution or hydrophobic microenvironment, $c_1/c_2 = (m_1 + e_1)/(m_2 + e_2)$ when c_1 and c_2 are the concentrations of pyrene-CE in the donor and acceptor at time t ($c_1 + c_2 = C$, total pyrene-CE in the incubation mixture). For the initial part of the reaction, $c_1/c_2 = (m_1 + e_1)/(m_2 + e_2) = m_1/m_2$ because $m >> e$. Therefore, $f = e_1/(m_1 + m_2) + e_2/(m_1 + m_2) = (e_1/m_1)(c_1/C) + (e_2/m_2)(c_2/C) = f_1(c_1/L_1) + f_2(c_2/L_2)$ when the lipid core concentration of donor particle is L_1, that of acceptor particle is L_2, and partial f values for donor and acceptor particles are f_1 and f_2, at incubation time t. Since $f_1 = a(c_1/L_1) + b$ and $f_2 = a(c_2/L_2 + b$, $f = f_1(c_1/C) + f_2(c_2/C) = a\{(c_1^2/L_1/C) + c_2^2/L_2/C)\} + b$. When $c = c_1 + c_2$ is substituted, $f = a(L_1 + L_2)c_1^2/L_1/L_2/C - 2ac_1/L_2 + aC/L_2 + b$. Under the experimental conditions, $L_2 >> L_1$ (therefore, $L_2 >> C$ and $L_2 >> c_1$) so that the equation is approximated as $f = a(c_1^2/L_1/C) + b$ for the initial part of the reaction (c_1/C is not far less than 1). Thus,

$$c_1 = L_1 C\,(f - b)/a \qquad (1)$$

when k is the average rate for the equilibrium transfer of total core lipid between the donor and acceptor particles, $-dc/dt = k(c_1/L_1 - c_2/L_2)$ assuming that the apparent rate constants of the transfer of TG and pyrene CE are approximately the same (Morton and Zilversmit, 1982; 1983). Then the integrated equation becomes $\ln \{L_2C/(L_1 + L_2)\} = -kt\,(L_1 + L_2)/L_1/L_2 + \ln \{L_2C/(L_1 + L_2)\}$. Because $L_2 >> L_1$, the equation is approximated as:

$$\ln c_1 = -(k/L_1)t + \ln C \qquad (2)$$

From equations (1) and (2),

$$\ln(f-b) = -(2k/L_1)t + \ln(aC/L_1) \quad (3)$$

Thus, the plot of logarithm of $(f-b)$ against incubation time allows one to obtain the rate constant k/L_1 from the slope.